Biological materials uses thereof

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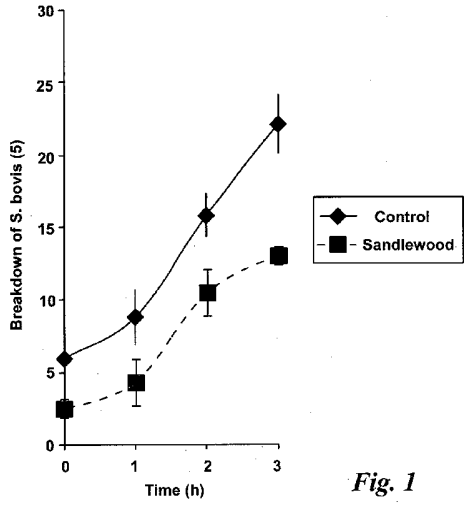


Fig. 1

(57) Abstract: There is provided the use of a sandalwood extract or a sandalwood analogue as an additive to animal foodstuff for the reduction of methane production, reduction of bacterial mediated protein breakdown and reduces bacterial growth in the stomach. There are also provided food products and methods of making food products incorporating sandalwood extracts or sandalwood analogues.

WO 2009/153572 A1

BIOLOGICAL MATERIALS AND USES THEREOF

The present invention relates to additives for animal foodstuffs and to methods for beneficially regulating ruminant digestion.

The supply of antibiotic growth promoters to farm animals is a well known method in agriculture for increasing the yield of meat or diary produce. The term "antibiotic growth promoter" is used to describe any medicine that destroys or inhibits bacteria and is administered at a low, sub-therapeutic dose. Infectious agents reduce the yield of farmed food animals and, to control these, the administration of sub-therapeutic antibiotics and antimicrobial agents has been shown to be effective. Although the mechanism underpinning their action is unclear, it is believed that the antibiotics suppress sensitive populations of bacteria in the intestines.

It has been estimated that as much as six percent of the net energy in the pig diet could be lost due to microbial fermentation in the intestine. If the microbial population could be better controlled, it is possible that the lost energy could be diverted to growth. Similarly upwards of ten percent of the energy in the diet of cattle and sheep is lost through the production of methane during microbial fermentation, decreasing methane production in the rumen using antimicrobial agents not only diverts this energy to meat and milk production but also lower the production of this harmful greenhouse gas. Whatever the mechanism of action, the use of growth promoters results in an improvement in daily growth rates between one and ten percent resulting in meat of a better quality, with less fat and increased protein content.

Currently, there is some unease surrounding the use of growth promoters in animals destined for meat production, as overuse of any antibiotic over a period of time may lead to the local bacterial populations becoming resistant to the antibiotic. Human health is potentially also directly affected through residues of an antibiotic in meat, which may cause side-effects.

In response to growing concerns regarding the effects of antibiotic growth promoters on human health, in January 2006 the European Union effectively prohibited on the use of antibiotics as growth promoters in animal agriculture. As such, there is currently an unsatisfied demand for alternatives to antibiotics. Livestock producers must find alternative means of obtaining similar production benefits to maintain and improve the standards and quantities of livestock products but also to maintain the profitability and

competitiveness of the livestock industry. Some countries around the world, including the USA, do not currently have restrictions on the use of antibiotics as growth promoters in animal agriculture, however such restrictions may exist in the near future and there is also a need to improve those livestock that are treated with antibiotics. Ways must also be found to improve the healthiness and safety of animal products reaching the consumer, including those from organic farming.

There are also important social issues concerning the removal of antibiotic growth promoters including possible higher cost of production being passed to the consumer and the risks to both human and animal health through the greater prevalence of pathogenic organisms in the animal. These factors will drive the rapid acceptance of new products, providing they are efficacious.

We have identified a plant extract (selected from a screening of almost 2500 such compounds) that beneficially manipulates digestion in the gut of ruminant livestock to promote the economic, safe and environmentally friendly production of meat and milk. Specifically the extract of interest prevents the growth of *E.coli* 0157 and *Listeria monocytogenes* in the rumen; reduces the rate of protein breakdown (allowing more protein to be absorbed by the gut of the animal and thus boosting production); and decreases the emission of the important greenhouse gas methane.

A first aspect of the invention provides the use of a sandalwood extract or a sandalwood analogue as an additive to animal foodstuff.

Sandalwood extract is an essential oil extracted from trees in the genus *Santalum*. The extract has commonly been used for incense, aromatherapy and as an ingredient in perfume. Sandalwood essential oil has also been used in medicine, mostly as a urogenital and skin antiseptic. Its main component, santalols, has known antimicrobial properties. However, it has not previously been suggested that sandalwood extract could beneficially manipulate ruminant digestion in the gut of ruminant livestock.

As disclosed herein, the inventors have determined that sandalwood extract prevents the growth of *E.coli* 0157 and *Listeria monocytogenes* in the digestive system of ruminants. In addition there is also a reduction in protein breakdown in the rumen, which allows more protein to be absorbed thus boosting meat and milk production, and a decrease in the emission of methane from the rumen. Therefore sandalwood extract can be used as

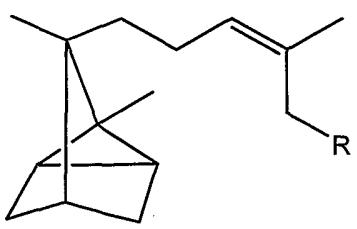
an additive to animal foodstuff in order to bring about important and beneficial changes in ruminant digestion.

It is important to note that the beneficial properties of sandalwood extract are not common to all extracts or compounds having antimicrobial properties. For example, during our trials we have tested some 2500 plant extracts including numerous essential oil compounds without finding a comparable extract.

By "sandalwood extract" we include where the extract is the essential oil prepared from trees of the genus *Santalum*. The extract can be obtained commercially from very many sources. Examples of sandalwood extract that can be used in the present invention include: Sandalwood oil manufactured by SAFC (e.g. W30,050-0 lot no. 03722CC-396) and Sandalwood oil manufactured by Fluka (355263/1 lot no. 52706264), Sandalwood oil from Swiss Herbal Remedies (B/N 540).

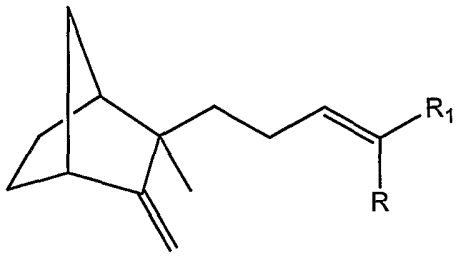
By "sandalwood analogue" we mean a compound or mixture of compounds that resembles sandalwood on the basis of smell (see, for example, Bieri et al (2004) Chem Senses. 29(6):483-7 Olfactory receptor neuron profiling using sandalwood odorants). Such analogues include natural analogues extracted from natural sources such as essential oils and synthetic sandalwood replacement compounds or mixtures. Examples of synthetic replacements include Javanol™ (e.g. from Givaudan lot nos. 9000591570 and 90000635339) and Santaliff™ (e.g. from International Flavour and Fragrances lot no. R000485362). Further alternatives are readily available and a number of these alternatives are discussed further in the examples.

Such compositions contain chemical compounds having the structures:



where:

α-Santalol : R = OH

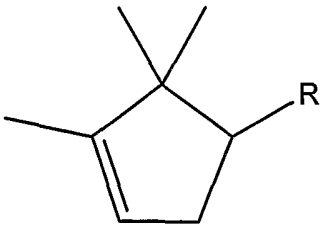


where

for β-Santalol : R = CH_2OH and R1 = H

for E-cis-epi-β-Santalol: R = H and R1 = CH_2OH

and for those chemical analogues based on campholenic aldehyde:



where:

R =3 methyl pentanol	=	Sandalore:
R=3-methyl pent-4-en-2-ol	=	Ebanol
R = (*E*)-2-methylbut-2-en-1-ol	=	Santaliff
R = (*E*)-2-ethylbut-2-en-1-ol	=	Bacdanol and Sanjinol isomers

Other R groups are possible, as will be apparent to those skilled in the art.

As discussed above, sandalwood extract or analogues thereof can be used to bring about these important and beneficial changes in ruminant digestion. Hence it is preferred that it used as an additive in ruminant diets althougth it may also be beneficial in monogastric animals such as horses.

A ruminant is an animal that digests its food in two steps: first by eating the raw material and regurgitating a semi-digested form known as a cud, then eating the cud, a process called ruminating. Ruminants have a stomach with four chambers, which are the rumen, reticulum, omasum and abomasum. In the first two chambers, the rumen and the reticulum, the food is mixed with saliva and separates into layers of solid and liquid

material. Solids clump together to form the cud (or *bolus*). The cud is then regurgitated, chewed slowly to completely mix it with saliva, which further breaks down fibers. Fibre, especially cellulose, is broken down into glucose in these chambers by *symbiotic* bacteria, protozoa and fungi. The broken-down fiber, which is now in the liquid part of the contents, then passes through the rumen into the next stomach chamber, the omasum, where water is removed. After this the digesting food is moved to the last chamber, the abomasum. The food in the abomasum is digested much like it would be in the human stomach. It is finally sent to the small intestine, where the absorption of the nutrients occurs.

Almost all the glucose produced by the breaking down of cellulose is used by the symbiotic bacteria. Ruminants get their energy from the volatile fatty acids produced by these bacteria: lactic acid, propionic acid and butyric acid.

Ruminant animals include include cattle, goats, sheep, camels, llamas, giraffes, bison, buffalo, deer, wildebeest and antelope. Preferably the sandalwood extract is used as an additive for foodstuffs for domesticated livestock such as cattle, goats, sheep or llamas.

The sandalwood extract or analogue can be added to the foodstuff after the foodstuff has been prepared or during preparation of the foodstuff.

Preferably the foodstuff is suitable for administration to an animal, particularly a ruminant or horse. Whilst note exclusive examples of foodstuffs to which the sandalwood extract can be added include: total mixed rations (TMR) ensiled and fresh forage, grains, manufactured concentrates protein supplement and by-products. However it is likely that the preferred method of addition would be via premixes and mineral and vitamin supplements either incorporated into diets of off or on farm.

The amount of sandalwood extract or analogue used in the invention is between 0.025 g per day and 50g per day. Preferred amounts are between 0.5 and 50g/day for larger ruminants e.g. cattle, preferably 5g/day. Further preferred amounts are between 0.025g and 2.5 g /day for small ruminants such as sheep preferably 0.25g/day.

These amounts can alternatively be expressed as 25mg/kg – 50g/kg, preferably 500mg/kg.

A further method of the invention provides a method for reducing the growth of pathogenic bacteria in the digestive system of a ruminant or horse comprising supplying the ruminant or horse with sandalwood extract or an analogue thereof.

As set out in the accompanying examples, sandalwood extract or an analogue thereof acts within the digestive system to reduce pathogenic bacterial growth. The reduction in pathogenic bacterial growth caused by the method of the invention is beneficial as there is also a reduction in bacterial levels in meat derived from ruminants. Since some bacteria pose significant hazards to human health, for example *E.coli*, then the method of the invention can be useful in improving the hygiene of meat. A preferred embodiment of this aspect of the invention is wherein bacterial growth is reduced in the rumen.

By "reducing" we include that the sandalwood extract reduces bacterial growth by 25% in comparison to a reference sample.

Preferably the method of this aspect of the invention reduces *E.coli* and/or *Listeria monocytogenes* growth.

Sandalwood extract or an analogue thereof is supplied to a ruminant or horse as part of the method of this aspect of the invention. Examples of sandalwood extract as an additive are described above and are suitable for use in the method of the invention. Preferably the method of this aspect of the invention uses the animal foodstuff set out above.

A further aspect of the invention provides a method of increasing meat and/or milk production from a ruminant or horse comprising supplying the ruminant or horse with sandalwood extract or an analogue thereof.

A still further method of the invention provides a method for reducing protein breakdown in the digestive system of a ruminant or horse comprising supplying the ruminant or horse with sandalwood extract or an analogue thereof.

By "increasing meat and/or milk production" we include that the sandalwood extract or an analogue thereof increases meat and/or milk production by at least 5- 10% of the weight or volume of the product , in comparison to a reference sample.

By "*reducing protein breakdown* in the digestive system" we include that the sandalwood extract or an analogue thereof reduces protein breakdown in the digestive system by 10 - 20% in comparison to a reference sample.

As set out in the accompanying examples, sandalwood extract or an analogue thereof acts within the digestive system to decrease in protein degradation in the gut and hence increase protein absorption by the animal. The increase in protein absorption leads to increased meat and/or milk production from the ruminant or horse and/or reduced feeding costs.

An embodiment of the method of the invention is wherein protein breakdown is reduced in the rumen.

A further method of the invention provides a method of reducing methane emission by a ruminant or horse comprising supplying the ruminant or horse with sandalwood extract or an analogue thereof.

Sandalwood extract or an analogue thereof can be supplied to a ruminant or horse as part of the methods of these aspects of the invention. Examples of sandalwood extract or an analogue thereof as an additive are described above and are suitable for use in these methods of the invention. Preferably these methods of the invention use the animal foodstuff set out above.

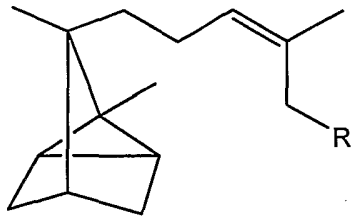
A further aspect of the invention provides the use of sandalwood extract or an analogue thereof to reduce the growth of pathogenic bacteria in the digestive system of a ruminant or horse.

A further aspect of the invention provides the use of sandalwood extract or an analogue thereof to increase meat and/or milk production from a ruminant or horse.

A further aspect of the invention provides the use of sandalwood extract or an analogue thereof to reduce protein breakdown in the digestive system of a ruminant or horse.

A further aspect of the invention provides the use of sandalwood extract or an analogue thereof to reduce methane emission by a ruminant or horse.

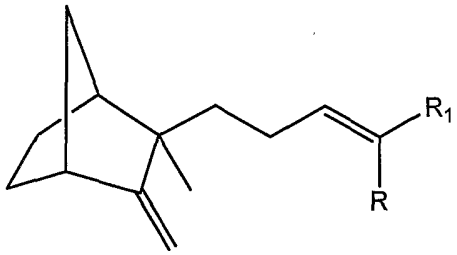
Preferably the sandalwood analogue of any aspect of the invention has the structure:



where:

R = OH

Alternatively, the sandalwood analogue of any aspect of the invention has the structure:

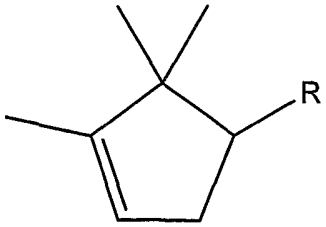


where

R = CH_2OH and R1 = H; or

R = H and R1 = CH_2OH

Further alternatively, the sandalwood analogue of any aspect of the invention has the structure:



where:

R =3 methyl pentanol, 3-methyl pent-4-en-2-ol, (*E*)-2-methylbut-2-en-1-ol, or (*E*)-2-ethylbut-2-en-1-ol

The invention will now be described in more detail, for the purposes of illustration only, in the following Examples and Figures.

Figure 1 - The effect of 500 µg/ml Sandalwood Oil on the breakdown of *S. bovis* protein in rumen fluid.

Figure 2 - Effect of Sandalwood Oil on the decline of E. coli O157 in the rumen simulation fermentor Rusitec

Figure 3 - Effect of Sandalwood Oil on the decline of Listeria monocytogenes in the rumen simulation fermentor Rusitec

Figure 4 - shows the results of the methane production, and demonstrates that the Sandalwood oil from Fluka and both batches of Javanol and Santalifff when compared to the control experiments significantly decreased methane

Figure 5 - Sandalwood oil and Javanol effect on methane production.

Figure 6 – Chemical structures of:

i) α-Santalol
(5-(2,3-dimethyl-tricyclo[2.2.1.02,6]hept-3-yl)-2-methyl-pent-2-en-1-ol)

ii) β-Santalol
(2-methyl-5-(2-methyl-3-methylene- bicyclo[2.2.1]hept-2-yl-pent-2-en-1- ol)

iii) α-Santalene
(1, 7-dimethyl-7-(4-methyl-3-pentenyl)-tricyclo[2.2.1.0(2,6)]heptane)

iv) *Z*-α-*trans*-β-Bergamotol
(1S-(1a.,5a.,6a.(Z)-5-(2,6-dimethylbicyclo(3.1.1)hept-2-en-6-yl)-2-methyl-2-penten-1-ol

v) *E*-*cis*,epi-β-Santalol

(2-methyl-5-((1R,2R,4S)-2-methyl-3 -methylenebicyclo(2.2.1)hept-2-yl)-(2Z)- 2-penten-1-ol)

vi) *cis*-Nuciferol

(S-(Z)-2-methyl-6-(4-methylphenyl)- 2-hepten-1-ol)

vii) Farnesol (*trans,trans*)

(E,E) 3,7,11-trimethyl-2,6,10-dodectrien-1-ol

Figure 7 – Chemical structures of:

i) Javanol[1]

(1-Methyl-2-(1,2,2-trimethylbicyclo[3.1.0]hex-3-ylmethyl)cyclopropyl)methanol

ii) Sandalore

5-(2,2,3-Trimethyl-3- cyclopentenhyl)- 3-methylpentan-2-ol

iii) Ebanol

3-Methyl-5-(2,2,3-trimethyl-3-cyclopenten-1-yl)-4-penten-2-ol

iv) Sandela[2]

4-(5,5,6-Trimethylbicyclo[2.2.1]hept-2-yl) cyclohexan-1-ol

Figure 8 – Chemical structures of:

(i) Santaliff

(2-Methyl-4-(2, 2, 3-trimethyl-3-cyclopenten-1-yl)-2-buten-1-ol

(ii) Bacdanol[1]

(2-ethyl-4-(2, 2, 3-trimethyl-3-cyclopenten-1-yl)-2-buten-1-ol)

(iii) Sanjinol[1]

(2-ethyl-4(2,2,3-trimethyl-3-cyclopentenyl) -2-buten-1-ol)

Figure 9 Sandalwood oil (Swiss Herbal Remedies)

Figure 10 - Sandalwood Oil (sample D, SAFC)

Figure 11 - Sandalwood Oil Sample (sample E, Fluka)

Figure 12 - Purity of chemical analogues by gc-ms: Javanol (sample A)

Figure 13 - Purity of chemical analogues by gc-ms: Javanol (sample B)

Figure 14 - Determination of Purity of Santaliff BHT by gc-ms (sample C)

Figure 15 - Purity of farnesol (trans,trans) standards by gc-ms

Example 1 - Effects of Sandalwood oil on methane production from extracted rumen fluid

An initial screening of Sandalwood oil for it effects on volatile fatty acid and methane production in the rumen was carried out.

Thirty ml of a 33% buffered solution of rumen fluid withdrawn from a rumen canulated cow was incubated with 0.3 g of a 50:50 hay barley mix at 39^{o}C under anaerobic conditions.

After 16 hours the volume of gas produced and the percentage of methane in the headspace was measured and the resultant fermentation fluid was analysed for volatile fatty acids by HPLC.

Sandalwood oil, and control oils of commercial essential oil mixes or pure oils of eugenol or cinnamaldehyde were added in the amount of 500 µg/ml to the 30ml buffered solution of rumen fluid prior to incubation. Sandalwood oil was obtained from Cardiff University and Sigma [SAFC (e.g. W30,050-0 lot no. 03722CC-396) and Sandalwood oil manufactured by Fluka (355263/1 lot no. 52706264).]

Table 1 shows the results of the methane production and HPLC analysis, and demonstrates that the Sandalwood oil when compared to the control experiments

significantly decreased methane production and stimulated propionate production at the expense of acetate formation.

Table 1 Effect of essential oils (500 µg/ml) on total volatile fatty acid and methane production by rumen fluid

		Acetate	Propionate	Butyrate	Total volatile fatty acids	Methane
	formation µ mole/ g of feed					
Cinnamaldehyde		2832	492	73	3398	1471
Eugenol		2817	590	73	3479	1436
Citral		2754	534	72	3361	1461
Essential oil mix 1		2816	479	72	3367	1455
Essential oil mix 2		2559	460	77	3095	1411
Essential oil mix 3		2727	485	97	3309	1480
Sandalwood oil		2266[a]	1083[a]	61	3410	1089[a]
Control		2768	519	75	3362	1446
Standard error difference (SED)		228.7***	99.1***	12.9***	211.3***	97.7***

"a" = Different substrates significantly differing from the mean

"***" = P>0.001

Example 2 - Influence of sandalwood oil on breakdown of labelled bacteria

In a further screen the ability of the Sandalwood oil to prevent the breakdown of bacteria by protozoa in the rumen was tested using the methods described by Wallace and McPherson (1987) *Br J Nutr.*, **58**, pp313-23. Briefly, the rumen bacterium *Streptococcus bovis* (*S.bovis*) was radio-labelled by growing it in a minimal media with ^{14}C- lysine as the only available nitrogen source.

The labelled *S. bovis* were washed and then incubated anaerobically in rumen fluid at 39°C for 3h in the presence or absence of 500 µg/ml Sandalwood oil. The release of C^{14} was monitored by liquid scintillation spectrometry.

Sandalwood oil caused a significant decrease in the breakdown of the labelled *S. bovis* suggesting that addition of Sandalwood oil reduced bacterial protein turnover in the rumen (Figure 1).

Example 3 - Rusitec simulation

Further investigations of the action of Sandalwood oil were undertaken in the rumen simulation technique Rusitec. Rusitec was developed by Czerkawski and colleagues (Czerkawski and Breckenridge (1977) Br J Nutr. 38 , 371-84) as a long term simulation of rumen fermentation and has been used extensively to test feed additive for ruminants.

Simulation 1

The rumen-simulation technique (Rusitec) was used as described by Czerkawski and Breckenridge (1977).

The nominal volume in each reaction vessel was 850 ml and the dilution rate was set at 0.88 per day, the infused liquid being artificial saliva (McDougall, Biochem J 1948 43, 99-109) at pH 8.4. Inocula for the fermentation vessels were obtained from a pooled sample (liquid and particulate rumen contents) from three rumen-cannulated cattle fed on a conserved diet.

On the first day of the experiment 300 ml of strained rumen fluid, 300 ml of water and 300 ml of artificial saliva were placed in each reaction vessel. Solid rumen contents (80 g) were weighed into a nylon bag and one of these was placed inside the food container in each vessel together with a bag (20g/d) of a basal diet of grass hay, barley, molasses, soyabean meal and a vitamin and mineral mixture. The food was provided in nylon bags, pore size 50 µm, which were gently agitated in the liquid phase. Two bags were present at any time and one bag was replaced each day to give a 48 h incubation.

The bags that were removed from the vessels were placed in plastic bags, and their contents washed and squeezed with 40 ml of artificial saliva. This was done twice for each bag, and the combined washings were poured back into the reaction vessels. Fermentation vessels were flushed with anaerobic grade CO_2 before filling, after filling, and then every day during feeding (when the nylon bags with the food were changed).

The duration of the experiment was 11 days, during which four vessels received Sandalwood oil (Fluka) (added the basal diet to reach an initial concentration of 333 µg/ml) the remaining vessels were controls.

Volatile fatty acids, ammonia and bacterial numbers were measured on days 10 and 11 of the experiment. On day 11 a non verotoxin containing strain of *E. coli* 0157 was added and it numbers traced over the last 24 hr of the experiment.

Simulation 2

In a second experiment a very similar protocol to simulation 1 was followed however 12 vessels were used and Sandalwood oil was added the basal diet to reach an initial concentrations of 0, 5, 50 or 500 µg/ml in triplicate vessels and the Sandalwood oil was sourced from Sigma Chemicals Ltd. The cattle used to provide the initial inoculum were grazing and a different source of hay and soyabean meal were used in the basal diet. The decline in the pathogen *Listeria monocytogenes* rather than *E. coli* 0157 was monitored.

Table 2 Effect of Sandalwood oil on daily volatile fatty acid production in the rumen simulation fermentor Rusitec

	Sandalwood oil concentration µg/ml		
	0	333	SED
Acetate mmol/d	16.2	15.1	0.90
Propionate mmol/d	12.2	12.1	0.92
Butyrate mmol/d	4.2	3.8	0.41
Total VFA mmol/d	32.6	30.9	2.10

Table 3 Effect of Sandalwood oil on daily ammonia and methane production and bacterial numbers in the rumen simulation fermentor Rusitec

	Sandalwood concentration µg/ml		
	0	333	SED
Vessel pH	6.8	6.8	0.03
NH_3 mmol/d	32.3	20.7	1.71
CH_4 mmol/d	1.0	0.77	0.131
24 h DM degradation %	21.5	22.4	6.70
48 h DM degradation %	35.1	35.4	6.03
Bacteria counts			
Total /ml	$5.0x10^9$	$6.0x10^9$	$0.26x10^9$
Log cellulolytic (Back transformed means / ml)	8.39 ($2.45x10^8$)	7.33 ($0.21x10^8$)	0.350

Table 4 Effect of Sandalwood oil on daily volatile fatty acid production in the rumen simulation fermentor Rusitec

	Sandalwood Oil concentration (ug/ml)				
	0	5	50	500	SED
Acetate mmol/d	26.5	23.1	23.9	25.9	3.60
Propionate mmol/d	23.5	22.6	23.0	30.2	3.269
Butyrate mmol/d	11.1	7.9	10.2	8.8	3.09
Total VFA mmol/d	65.3	60.0	60.5	78.0	13.32

Table 5 Effect of Sandalwood oil on daily ammonia and methane production and bacterial numbers in the rumen simulation fermentor Rusitec

	Sandalwood Oil concentration (ug/ml)				
	0	5	50	500	SED
Vessel pH	6.7	6.7	6.7	6.7	0.05
NH_3 mmol/d	439	511	458	138	118.3
CH_4 mmol/d	4.5	2.7	2.4	2.1	0.35
24 h degradation %	16.5	23.5	15.4	18.5	9.29
48 h degradation %	38.6	32.7	26.7	30.0	5.44
Bacteria counts					
Total /ml	$1x10^9$	$1x10^9$	$2x10^9$	$3x10^9$	$9.4x10^8$
Cellulolytic /ml	$8.6x10^6$	$2.3 x10^6$	$8.7 x10^6$	$4.0 x10^6$	$3.49x10^6$

Sandalwood oil had no effect on VFA production in either experiment (Tables 2 and 4). Ammonia production was reduced by Sandalwood oil added at either 333 or 500 µg/ml but not lower concentrations (Tables 3 and 5).

Methane production was decreased by all concentration above 5µg/ml (Tables 3 and 5). In the 1st experiment 333 µg/ml of Sandalwood oil significantly reduced the survival of *E. coli* 0157 in the fermentor (Figure 2) whilst in the second experiment Sandalwood oil at 50 or 500 µg/ml significantly reduced *Listeria monocytogenes* survival at 24h after pathogen addition (Figure 3).

Therefore, at between 50 and 500 µg/ml Sandalwood oil significantly reduced the production of methane an important greenhouse gas and also major energy loss from the animal. At concentrations above 333 µg/ml Sandalwood oil significantly decreased ammonia production suggesting a protein sparing effect. Sandalwood oil also significantly reduced the ability of the pathogens *E.coli* 0157 and *Listeria monocytogenes* to survive in the fermentor.

Example 4 – Methane reduction in response to Sandalwood oil and synthetic sandalwood oil replacements

Thirty ml of a 33% buffered solution of rumen fluid withdrawn from a rumen canulated cow was incubated with 0.3 g of a 50:50 hay barley mix at 39^{o}C under anaerobic conditions

After 16 hours the volume of gas produced and the percentage of methane in the headspace was measured and the resultant fermentation fluid was analysed for volatile fatty acids by HPLC.

Sandalwood oil from either Fluka or SAFC and two different batches of Javanol (Givaudan) or a single batch of Santaliff (International Flavors & Fragrances) (both Javanol and Santaliff are artifical Sandalwood replacements) were added in the amount of 5, 50 or 100 or 500 µg/ml to the 30ml buffered solution of rumen fluid prior to incubation.

Figure 4 shows the results of the methane production, and demonstrates that the Sandalwood oil from Fluka and both batches of Javanol and Santalifff when compared to the control experiments significantly decreased methane .

Example 5 – Rusitec experiments using Sandalwood oil and synthetic sandalwood oil replacements

The rumen-simulation technique (Rusitec) was used as described by Czerkawski and Breckenridge (1977).

The nominal volume in each reaction vessel was 850 ml and the dilution rate was set at 0.88 per day, the infused liquid being artificial saliva (McDougall, Biochem J 1948 43, 99-109) at pH 8.4. Inocula for the fermentation vessels were obtained from a pooled sample (liquid and particulate rumen contents) from three rumen-cannulated cattle fed on a conserved diet.

On the first day of the experiment 300 ml of strained rumen fluid, 300 ml of water and 300 ml of artificial saliva were placed in each reaction vessel. Solid rumen contents (80 g) were weighed into a nylon bag and one of these was placed inside the food container in each vessel together with a bag (20g/d) of a basal diet of grass hay, barley, molasses,

soyabean meal and a vitamin and mineral mixture. The food was provided in nylon bags, pore size 50 µm, which were gently agitated in the liquid phase. Two bags were present at any time and one bag was replaced each day to give a 48 h incubation.

The bags that were removed from the vessels were placed in plastic bags, and their contents washed and squeezed with 40 ml of artificial saliva. This was done twice for each bag, and the combined washings were poured back into the reaction vessels. Fermentation vessels were flushed with anaerobic grade CO_2 before filling, after filling, and then every day during feeding (when the nylon bags with the food were changed).

The duration of the experiment was 19 days, during which four vessels received Sandalwood oil (added the basal diet to reach an initial concentration of 100 µg/ml) four vessels received Javanol (added the basal diet to reach an initial concentration of 100 µg/ml) the remaining vessels were controls.

Volatile fatty acids, methane and bacterial numbers were measured on days 18 and 19 of the experiment. On day 19, *Listeria inocula* was added and it numbers traced over the last 24 hr of the experiment.

Table 6

	Control	**Javanol**	**Sandalwood**	**SED**
pH	6.70	6.66	6.67	0.022
CH_4 mmol/d	3.42	2.51	2.36	0.365
24 h DM degradation (5	21.7	24.7	23.9	3.47
48 g DM degradation (%)	28.4	33.6	31.8	5.10
Bacteria counts				
Total $x10^9$/ml	6.1	4.9	7.6	2.69
Cellulolytic $x10^6$/ml	1.6	1.9	1.8	0.25

Table 7

	Control	Javanol	Sandalwood	SED
Acetate mmol/d	21.2	23.9	21.4	2.70
Propionate mmol/d	14.5	16.7	13.4	1.86
Butyrate mmol/d	5.8	4.4	4.6	1.89
Total VFA mmol/d	41.5	45.0	39.4	2.83

Sandalwood oil and Javanol had no effect on VFA production .Methane production was decreased by both Sandalwood oil and Javanol. Sandalwood oil but not Javanol tended ($P>0.08$) to decrease *Listeria monocytogenes* survival at 24h after pathogen addition (Figure 5).

Example 6 – Chemical analysis of sandalwood oils and synthetic sandalwood oil replacements used in examples

Materials and Methods

Plant extracts and chemical analogues

The sandalwood oil used was sourced from Swiss Herbal Remedies (B/N 540), Sigma-Aldrich Fine Chemicals Limited (SAFC) and Fluka (Dorset).

Chemical analogues based on the chemical structure of β-santalol, include Santaliff™ (supplier International Flavour and Fragrances, lot no: R000485362) and Javanol™ (supplier Givaudan, lot no's: 9000591570 and 90000635339) - see figures 7 and 8 for their chemical structures.

Farnesol (*trans,trans*) was purchased from Sigma-Aldrich (Poole, Dorset).

Sample Preparation

Sandalwood Oils

A 3 µl aliquot of sandalwood oil was transferred to a glass container and 1ml of absolute ethanol added. The mixture was vortex mixed for one minute. A 100 µl aliquot of this mixture was transferred to another glass container and 300 µl of ethanol added, yielding a final sandalwood concentration of 0.075% v/v

Chemical analogues

A 5ul aliquot of sample was transferred to a glass container and 5ml of absolute ethanol added giving a final concentration of 0.1%v/v.

Farnesol standards

3 µl of farnesol was transferred to a glass container containing 1ml of absolute ethanol added and vortex mixed for one minute yielding a final concentration of 0.3% v/v.

Gas Chromatography-Mass Spectrometry (GC-MS)

Samples were analysed using an Agilent Technologies 6890N gas chromatograph equipped with an Agilent 5973 Network mass selective detector and an Agilent 7683 series autosampler. A non-polar Phenomenex ZB5MS fused silica capillary column (supplier: Phenomenex) was used with the following dimensions: 30m x 0.25mm id. and 0.25µm film thickness.

The oven temperature was programmed from 50°C to 240°C at a rate of 3°C min^{-1} and maintained at this final temperature for five minutes. The helium carrier gas was set at a flow-rate of 0.6ml min^{-1} and maintained under constant pressure. The injector, source and mass transfer lines were set at temperatures of 250°C, 230°C and 280°C respectively.

The mass detector was used in the positive electron impact ionisation mode (EI+) using an ionisation voltage of 70 eV. A scan range of 35 to 450 mass units was used for acquiring the mass spectra data with a sampling time of 2 which corresponds to 3.5 scans per second. Data acquisition was performed using the MSD Chemstation™

computer software. The injection port was configured for on-column Injections, hence, low sample volumes (0.2 µL) were used for all test samples and injected in the splitless mode. An ethanol solvent wash was included between sample injections and a solvent delay of three minutes applied to the mass detector.

The identification of the individual peaks were made by:

i) Comparing sample mass spectra to those stored in the NIST library database and

ii) Comparing sample mass spectra to published literature values.

The NIST libraries contain over 54,000 spectra. A reverse fit method was used for identification throughout. This method normalises data to 1000, hence compounds with library fits greater than 900 have a very high likelihood of being correctly assigned.

Table 8 - Sandalwood oil (Swiss Herbal Remedies brand)

Peak No.	RT (Mins)	Compound	Library Fit (Reverse)	Peak Area	% Peak Area
1	30.47	α-Santalene	961	217084066	0.60
	31.07	α-Bergamotene	968	71342373	0.19
2	31.71	epi-β-Santalene	954	122464940	0.33
	32.24	β-Santalene	953	214940582	0.59
	33.20	Curcumene	980	216370397	0.59
3	36.92	Dendrolasin	920	585451408	1.62
4	41.51	α-Santalol	948	7152370863	19.79
5	41.96	α-trans-Bergamotol	935	3809882649	10.54
6	42.4	epi-β-Santalol	939	62990558	1.91
7	43.12	trans-β-Santalol*	902	7289614401	20.17
8	43.49	Nuciferol	933	4408086672	12.19
9	44.48	Nuciferol isomer	833	1658581138	4.58
10	44.71	cis-Lanceol	927	1083461495	2.99
11	50.68	Unknown	-	991883190	2.74
			Total Area	36138975840	
			Compounds Identified (%)		74.47
			Total alcohol content (%)		41.87

Total alcohol content expressed as santalol is **41.87 %**

Table 9 - Sandalwood Oil (SAFC brand)

Peak No.	RT (Mins)	Compound	Library Fit (Reverse)	Peak Area	% Peak Area
1	30.46	α-Santalene	964	176,600,110	0.66
	31.06	α-Bergamotene	966	27,925,579	0.10
2	31.70	epi-β-Santalene	945	214,178,420	0.80
3	32.24	β-Santalene	963	323,842,042	1.21
	33.20	Curcumene	969	40,191,154	0.15
4	41.68	α-Santalol	947	13,388,942,510	50.12
5	41.99	α-trans-Bergamotol	952	1,726,157,246	6.46
6	42.53	epi-β-Santalol	950	1,385,388,247	5.186
7	43.21	trans-β-Santalol	953	7,344,111,142	27.49
	43.31	Nuciferol	941	trace	-
8	43.83	Santalol isomer	967	527,459,686	1.97
9	44.02	Unknown	-	373576932	1.39
10	44.58	cis-Lanceol	966	452,046,537	1.69
			Total Area	26,712,032,225	
			Compounds Identified (%)		95.83
			Total alcohol content (%)		84.77

Total alcohol content expressed as santalol is **84.77%**

Table 10 - Sandalwood Oil (Fluka brand)

Peak No.	RT (Mins)	Compound	Library Fit (Reverse)	Peak Area	% Peak Area
1	30.46	α-Santalene	967	158,399,943	1.07
	31.07	α-Bergamotene	968	27,597,223	0.18
2	31.70	epi-beta-Santalene	950	170,472,890	1.15
3	32.24	β-Santalene	959	258,738,060	1.75
4	33.11	Unknown	-	171,605,164	1.16
	33.22	Curcumene	894	Trace	-
5	41.53	α-Santalol	942	7,926,340,551	53.83
6	41.87	α-trans-Bergamotol	949	804,435,867	5.46
7	42.41	epi-β-Santalol	953	679,521,124	4.61
8	43.04	trans-β-Santalol	960	3,189,548,425	21.66
9	43.19	Nuciferol	949	197,251,727	1.34
	43.51	Santalol isomer	822	42,100,101	0.28
10	43.72	Santalol isomer	968	168,818,533	1.14
11	44.54	cis-Lanceol	961	129,590,895	0.88
12	*50.91	Diisoctyl phthalate (plasticizer impurity)	951	-	-
			Total Area	14,725,166,618	
			Compounds Identified (%)		93.35
			Total alcohol content (%)		81.52

Total alcohol content expressed as santalol is 81.52%.

Table 11 - Purity of chemical analogues by gc-ms: Javanol (sample A)

Peak No.	RT (Mins)	Compound	Peak Area	% Peak Area
1	38.00	Javanol Isomer 2	791441868	**40.82**
2	38.44	Javanol Isomer 2	1124046450	**57.98**
3	42.268	Unknown	22946317	1.18
		Total Area	1938434635	

Total % Purity = 98.62%

Table 12 - Purity of chemical analogues by gc-ms: Javanol (sample B)

Peak No.	RT (Mins)	Compound	Peak Area	% Peak Area
1	37.95	Javanol Isomer 1	406015504	37.63
2	38.41	Javanol Isomer 2	651570188	60.39
3	42.27	Unknown 1	13370100	1.23
4	42.88	Unknown 2	7962910	0.73
		Total Area	1078918703	

Total % Purity = 98.02%

Table 13 - Determination of Purity of Santaliff BHT by gc-ms (sample C)

Peak No.	RT (Mins)	Compound	Library Fit (Reverse)	Peak Area	% Peak Area
1	32.01	Unknown		16811049	0.72%
2	32.37	Santaliff isomer		31247462	1.35%
3	33.22	Santaliff	-	2238211688	96.80%
4	34.08	Butylated hydroxy toluene*	894	25731644	1.11%
		Total Area		2312001843	

Total % Purity = 98.15%

Summary of Results

The santalol isomers were found to be the major chemicals present in the sandalwood oil obtained from India and Indonesia, showing a total santalol content between 78.1 – 84.7 %.

The sandalwood oil procured from the Asia-Pacific/Australia region contained much lower amounts of santalol (mean 41.9%) with substantially high levels of α-trans-Bergamotol (mean 10.4%) and nuciferol (mean 12.6%) compared to sandalwood oil samples obtained from India and Indonesia. Oil from these latter two countries contained α-trans-Bergamotol and nuciferol at lower levels (5.4-7.6%) and (1.3-1.6%) respectively.

The proportion of the two major santalol isomers (α and β), in the various sandalwood oils, varied greatly and favoured the α-isomer in sandalwood oil samples from India and Indonesia (approximately 2:1), whilst slightly favouring the β -isomer for the oils obtained from the Asia-Pacific /Australia region (mean 0.95: 1).

The sandalwood oil procured from the Asia-Pacific/Australia region (Swiss Herbal Remedies) was the only oil found to contain the furano-sesquiterpene dendrolasin.

The chemical analogues santaliff and javanol showed a mean purity of 98.15% and 98.32%, respectively, when analysed by gc-ms.

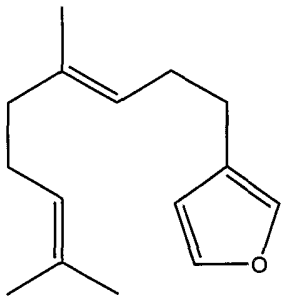
Analysis of the farnesol standards (old and new) showed the presence of the *cis,trans* farnesol isomer to substantially increase with time. Both farnesol standards showed greater than 96% purity for the sum of isomers.

Discussion of Results

All the sandalwood oils, tested, contained the same general bouquet of chemicals known as sesquiterpenes. These comprised of α-santalol, β-santalol, bergamotol, epi-santalol, nuciferol and lanceol. Their abundance in sandalwood oil was found to vary and depended upon the geographical location from which the oils were sourced.

GC-MS was able to classify the sandalwood oils into two chemical groups, according to their santalol content. Those oils containing high total santalol levels (Fluka and SAFC) and can be assured to be authentic sandalwood oil from the species S. *album*

The low santalol level and high nuciferol content detected for the oil from Swiss Herbal Remedies, however, suggests its origin to be either from *S. spicatum*, a species of sandalwood indigenous to Western Australia or *S. austrocaledonicum* from the Pacific Islands. *S. spicatum* has been reported to contain high a farnesol content (Jirovetz *et al.*, 2006), which was not confirmed in these oils. It is possible, however, that the farnesol peak (retention time 42 -43 minutes) was co-eluting with the β-santalol peak (42.8 -43 minutes). Repeat analysis on a more polar chromatography column would probably separate these compounds and confirm its presence. The detection of dendrolasin in the two samples of sandalwood oil labelled Swiss Herbal Remedies (Asia Pacific/Australia region) confirmed their origin to be from the sandalwood species *S. spicatum* indigenous to Australia.



Dendrolasin

Example 7 – Effect of further sandalwood analogues

Further testing of sandalwood analogues was conducted following the protocols described in Examples 3 and 5.

The sandalwood analogues (chemical structures are shown in figures 7 and 8) tested were:

Sanjinol
Bacdanol
Santaliff
Sandela
Javanol
Ebanol

Sandalore

Javanol (batch no. 9000699712), Sandalore (batch no. 9000703989), Ebanol (batch no. 900068333) and Sandela (batch no. 9000701064) were obtained from either Givaudan (UK) (via S. Black Limited, Hertford, UK) and Santaliff, Bacdanol and Sanjinol were obtained from International Flavour and Fragrances (Haverhill, UK)

Results

Table 14

Compound	µg/ml	Acetate	Propionate	Butyrate	TVFA	Methane
			µmol/g of feed added			
Sanjinol	5	1978	426	448	2852	1107
Sanjinol	50	2192	579	466	3237	835
Sanjinol	100	2359	697	512	3568	795
Sanjinol	500	1849	744	225	2818	741
Bacdanol	5	2037	444	438	2919	934
Bacdanol	50	2049	434	458	2942	1047
Bacdanol	100	2419	515	514	3448	995
Bacdanol	500	2331	864	309	3503	942
Santaliff	5	2174	466	458	3098	1031
Santaliff	50	2142	442	490	3074	1052
Santaliff	100	2301	543	462	3305	844
Santaliff	500	2101	741	296	3138	801
Sandela batch no. 9000701064	5	2223	465	482	3169	996
Sandela batch no. 9000701064	50	2235	450	513	3198	1052
Sandela batch no. 9000701064	100	2297	477	515	3289	1078
Sandela batch no. 9000701064	500	2270	680	317	3267	928
Javanol batch no. 9000699712	5	2401	493	538	3432	978
Javanol batch no. 9000699712	50	2622	524	593	3739	904
Javanol batch no. 9000699712	100	2504	542	499	3545	859
Javanol batch no. 9000699712	500	2045	833	229	3107	744
Ebanol batch no. 9000683337	5	2136	427	488	3051	952
Ebanol batch no. 9000683337	50	2129	436	474	3039	934
Ebanol batch no. 9000683337	100	2430	533	458	3421	833
Ebanol batch no. 9000683337	500	2333	939	332	3604	683
Sandalore batch no. 9000703989	5	2118	431	487	3036	951
Sandalore batch no. 9000703989	50	1972	409	453	2834	1086
Sandalore batch no. 9000703989	100	2119	471	432	3023	1174
Sandalore batch no. 9000703989	500	2249	897	305	3451	902
Control		1940	526	477	2943	1054
SED		198.7	49.33**	40.93	270.1	83.9***

Table 15

BOTTLE NO.	rep	Compound no.	compound rate ug/30ml	CH4 umol/g	
1	a	200ul ETOH	0	1153.037	
2	b	200ul ETOH	0	1341.099	
3	c	200ul ETOH	0	1034.304	1176.147
4	a	200ul DMSO	0	968.2334	
5	b	200ul DMSO	0	951.8643	
6	c	200ul DMSO	0	927.0059	949.0346
7	a	Javanol batch no. 9000699712 in 200 ul DMSO	150	956.9591	
8	b	Javanol batch no. 9000699712 in 200 ul DMSO	150	888.4071	
9	c	Javanol batch no. 9000699712 in 200 ul DMSO	150	895.6242	913.6635
10	a	Javanol batch no. 9000699712 in 200 ul DMSO	15000	647.283	
11	b	Javanol batch no. 9000699712 in 200 ul DMSO	15000	563.0216	
12	c	Javanol batch no. 9000699712 in 200 ul DMSO	15000	605.5467	605.2838
13	a	Javanol batch no. 9000699712 in 200ul ETOH	150	1403.051	
14	b	Javanol batch no. 9000699712 in 200ul ETOH	150	1381.609	
15	c	Javanol batch no. 9000699712 in 200ul ETOH	150	1335.905	1373.522
16	a	Javanol batch no. 9000699712 in 200ul ETOH	15000	1145.346	
17	b	Javanol batch no. 9000699712 in 200ul ETOH	15000	1230.103	
18	c	Javanol batch no. 9000699712 in 200ul ETOH	15000	1099.314	1158.255
19	a	Sanjinol in 200 ul DMSO	150	1107.671	
20	b	Sanjinol in 200 ul DMSO	150	793.8945	
21	c	Sanjinol in 200 ul DMSO	150	914.1195	938.5618
22	a	Sanjinol in 200 ul DMSO	15000	615.569	
23	b	Sanjinol in 200 ul DMSO	15000	893.1559	
24	c	Sanjinol in 200 ul DMSO	15000	722.2742	743.6664
25	a	Sanjinolin 200ul ETOH	150	1064.879	
26	b	Sanjinolin 200ul ETOH	150	1232.288	
27	c	Sanjinolin 200ul ETOH	150	1162.641	1153.269
28	a	Sanjinolin 200ul ETOH	15000	1063.899	
29	b	Sanjinolin 200ul ETOH	15000	1351.649	
30	c	Sanjinolin 200ul ETOH	15000	1059.708	1158.419

The sandalwood analogues tested (tables 14 and 15) showed a generalised improvement in a variety of properties when administered to the rusitec model. In particular, reductions in methane were achieved and these were shown to be dose specific reductions, such that increased doses of the sandalwood analogue are generally associated with a decrease in methane and volatile fatty acid production.

CLAIMS

1. The use of a sandalwood extract or a sandalwood analogue as an additive to animal foodstuff.

2. The use of Claim 1 wherein the extract or analogue is added to the foodstuff after the foodstuff has been prepared.

3. The use of Claim 1 wherein the extract or analogue is added to the foodstuff during preparation of the foodstuff.

4. The use of any one of the previous claims wherein the amount of sandalwood extract or analogue used is 0.025 and 50 g per day.

5. The use of claim 4 wherein the amount is between 0.5 and 50 g per day for large ruminants and horses and 0.025 g – 2.5 g per day in smaller ruminants.

6. The use of claim 5 wherein the amount is 5g per day for large ruminants and horses.

7. The use of claim 5 wherein the amount is 2.5g per day for small ruminants

8. The use of any one of the claims 1 to 3 wherein the amount of sandalwood extract or analogue used is between 25mg/kg – 50g/kg.

9. The use of claim 8 wherein the amount is 500mg/kg.

10. An animal foodstuff comprising a sandalwood extract or a sandalwood analogue.

11. The foodstuff of Claim 10 comprising between 0.025 and 50 g per day of sandalwood extract or analogue.

12. The foodstuff of claim 11 wherein the amount is between 0.5 and 50 g per day for large ruminants and horses and 0.025 g – 2.5 g per day in smaller ruminants.

13. The foodstuff of claim 12 wherein the amount is 5g per day for large ruminants and horses.

14. The foodstuff of claim 12 wherein the amount is 2.5g per day for small ruminants

15. The foodstuff of any one of the claims 1 to 3 wherein the amount of sandalwood extract used is between 25mg/kg – 50g/kg.

16. The foodstuff of claim 8 wherein the amount is 500mg/kg.

17. The foodstuff of any of claims 10 to 16 packaged and presented for feeding a ruminant or a horse.

18. A method for reducing the growth of pathogenic bacteria in the digestive system of a ruminant or horse comprising supplying the ruminant or horse with a sandalwood extract or a sandalwood analogue.

19. The method of Claim 18 wherein pathogenic bacterial growth is reduced in the rumen.

20. The method of Claim 18 or 19 wherein the bacteria is *E.coli* and/or *Listeria monocytogenes*.

21. The method of any of Claims 18 to 20 wherein the ruminant or horse is supplied with the animal foodstuff of Claims 10 to 17.

22. A method of increasing meat and/or milk production from a ruminant or horse comprising supplying the ruminant or horse with a sandalwood extract or a sandalwood analogue.

23. The method of Claim 22 wherein the ruminant or horse is supplied with the animal foodstuff of Claims 10 to 17.

24. A method for reducing protein breakdown in the digestive system of a ruminant or horse comprising supplying the ruminant or horse with sandalwood extract.

25. The method of Claim 24 wherein protein breakdown is reduced in the rumen.

26. The method of Claim 24 or 25 wherein the ruminant or horse is supplied with the animal foodstuff of Claims 10 to 17.

27. A method of reducing methane emission by a ruminant or horse comprising supplying the ruminant or horse with sandalwood extract.

28. The method of Claim 27 wherein the ruminant or horse is supplied with the animal foodstuff of Claims 10 to 17.

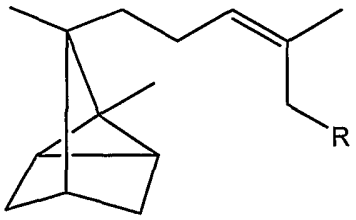
29. The use of a sandalwood extract or a sandalwood analogue to reduce the growth of pathogenic bacteria in the digestive system of a ruminant or horse.

30. The use of a sandalwood extract or a sandalwood analogue to increase meat and/or milk production from a ruminant or horse.

31. The use of a sandalwood extract or a sandalwood analogue to reduce protein breakdown in the digestive system of a ruminant or horse.

32. The use of a sandalwood extract or a sandalwood analogue to reduce methane emission by a ruminant or horse.

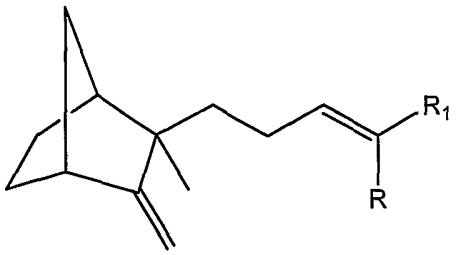
33. The use, foodstuff or method of any previous claim in which the sandalwood analogue has the structure:



where:

R = OH

34. The use, foodstuff or method of any previous claim in which the sandalwood analogue has the structure:

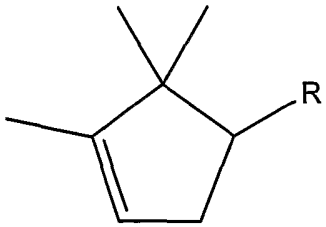


where

R = CH_2OH and R1 = H; or

R = H and R1 = CH_2OH

35. The use, foodstuff or method of any previous claim in which the sandalwood analogue has the structure:



where:

R =3 methyl pentanol, 3-methyl pent-4-en-2-ol, (*E*)-2-methylbut-2-en-1-ol, or (*E*)-2-ethylbut-2-en-1-ol

36. A use substantially as described herein with reference to the figures and examples.

37. A foodstuff substantially as described herein with reference to the figures and examples.

38. A method substantially as described herein with reference to the figures and examples.

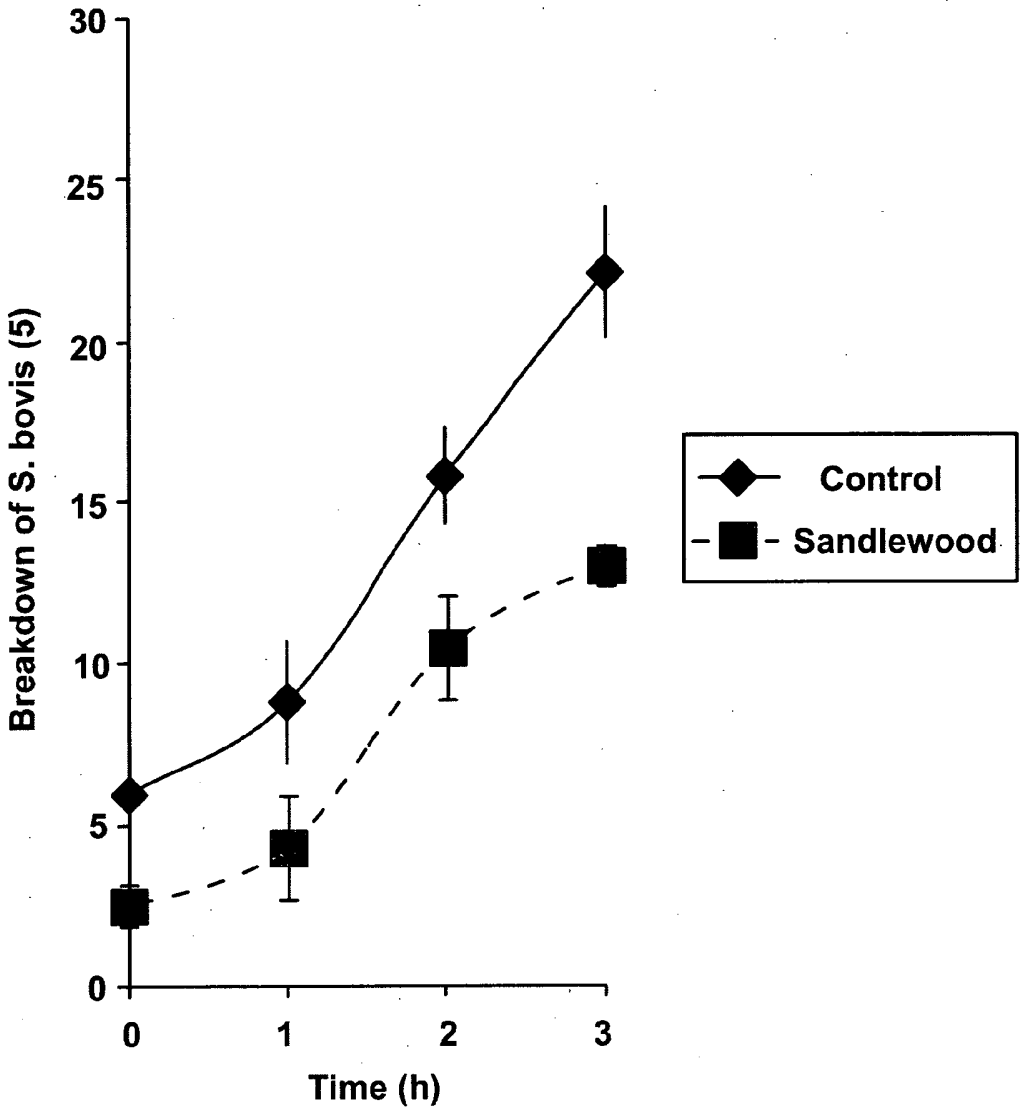

30
25
20
15
10
5
0
Breakdown of S. bovis (5)
0
1
2
3
Time (h)
Control
Sandlewood

Fig. 1

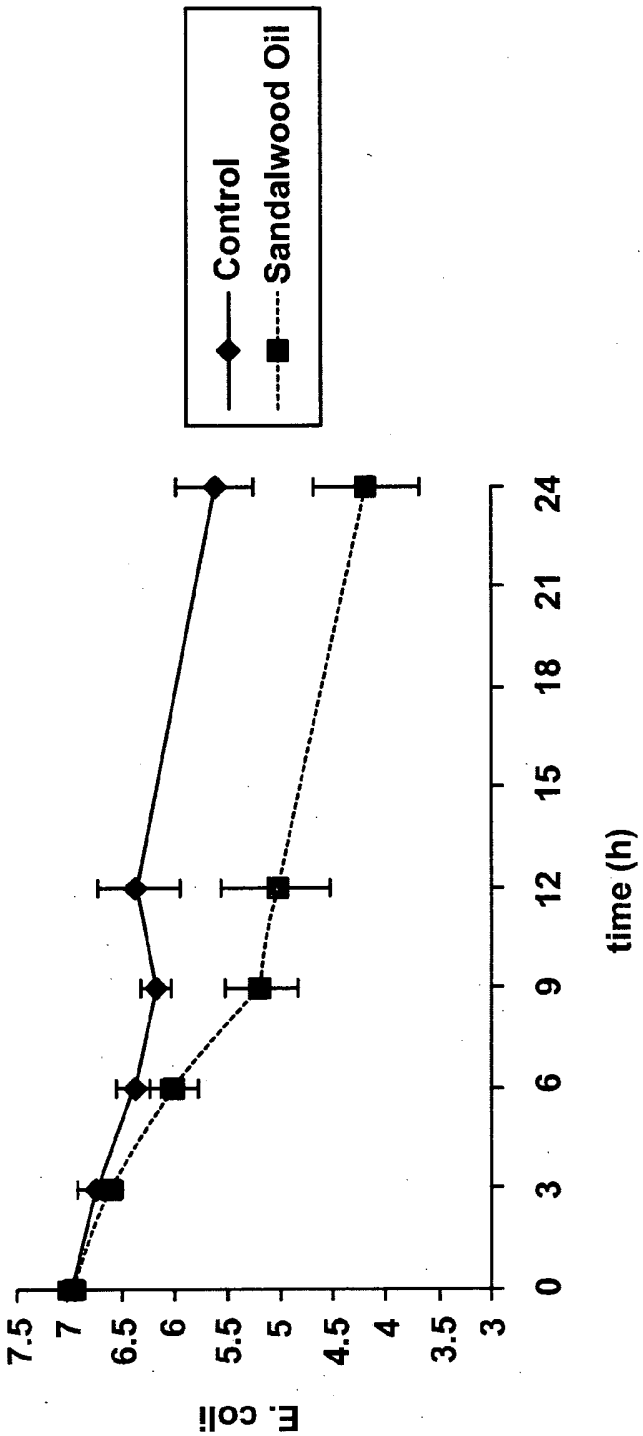

Control
Sandalwood Oil
E. coli
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
0
3
6
9
12
15
18
21
24
time (h)

Fig. 2

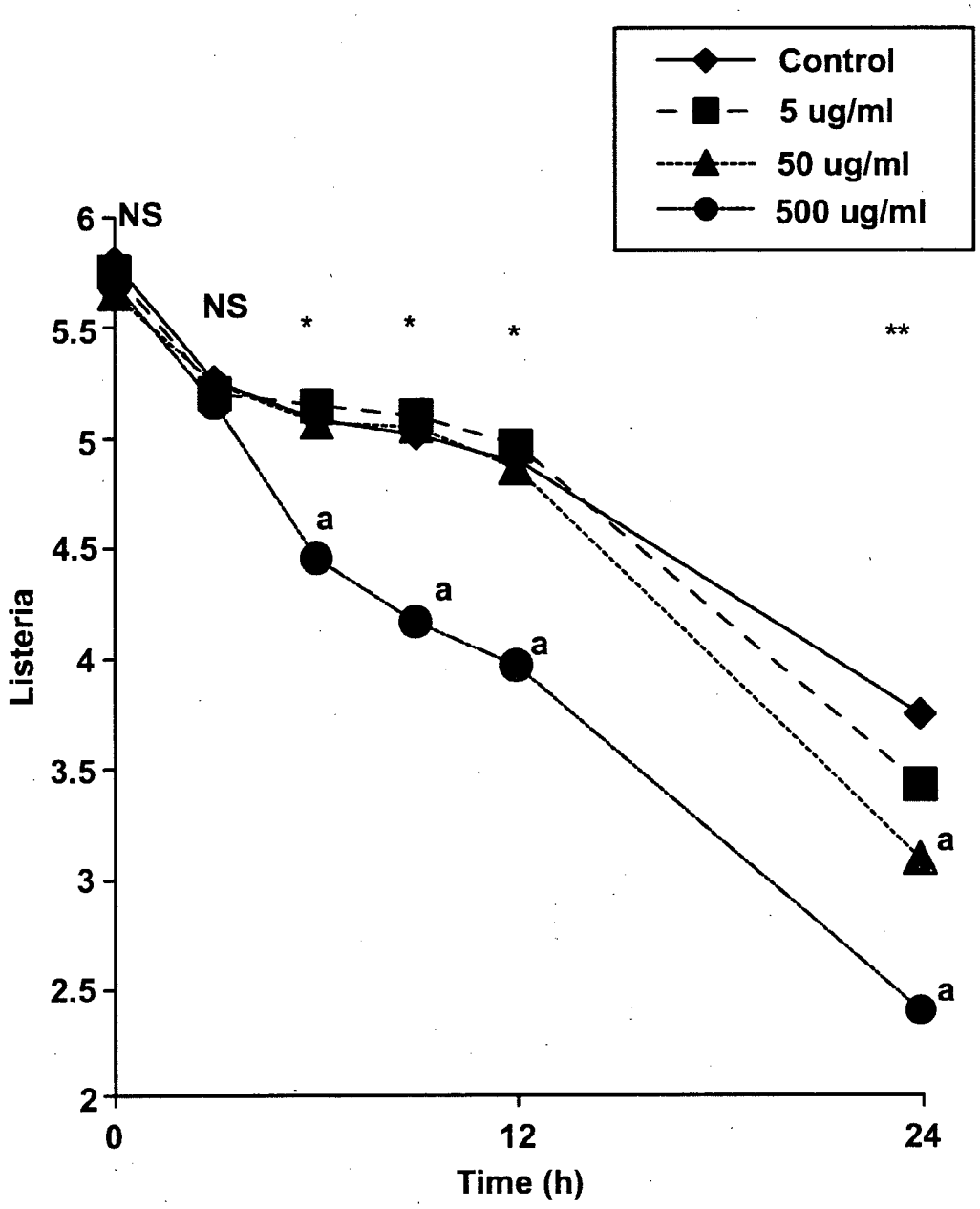

Control
5 ug/ml
50 ug/ml
500 ug/ml
Listeria
Time (h)
6
5.5
5
4.5
4
3.5
3
2.5
2
0
12
24
NS
NS
*
*
*
**
a
a
a
a
a

Fig. 3

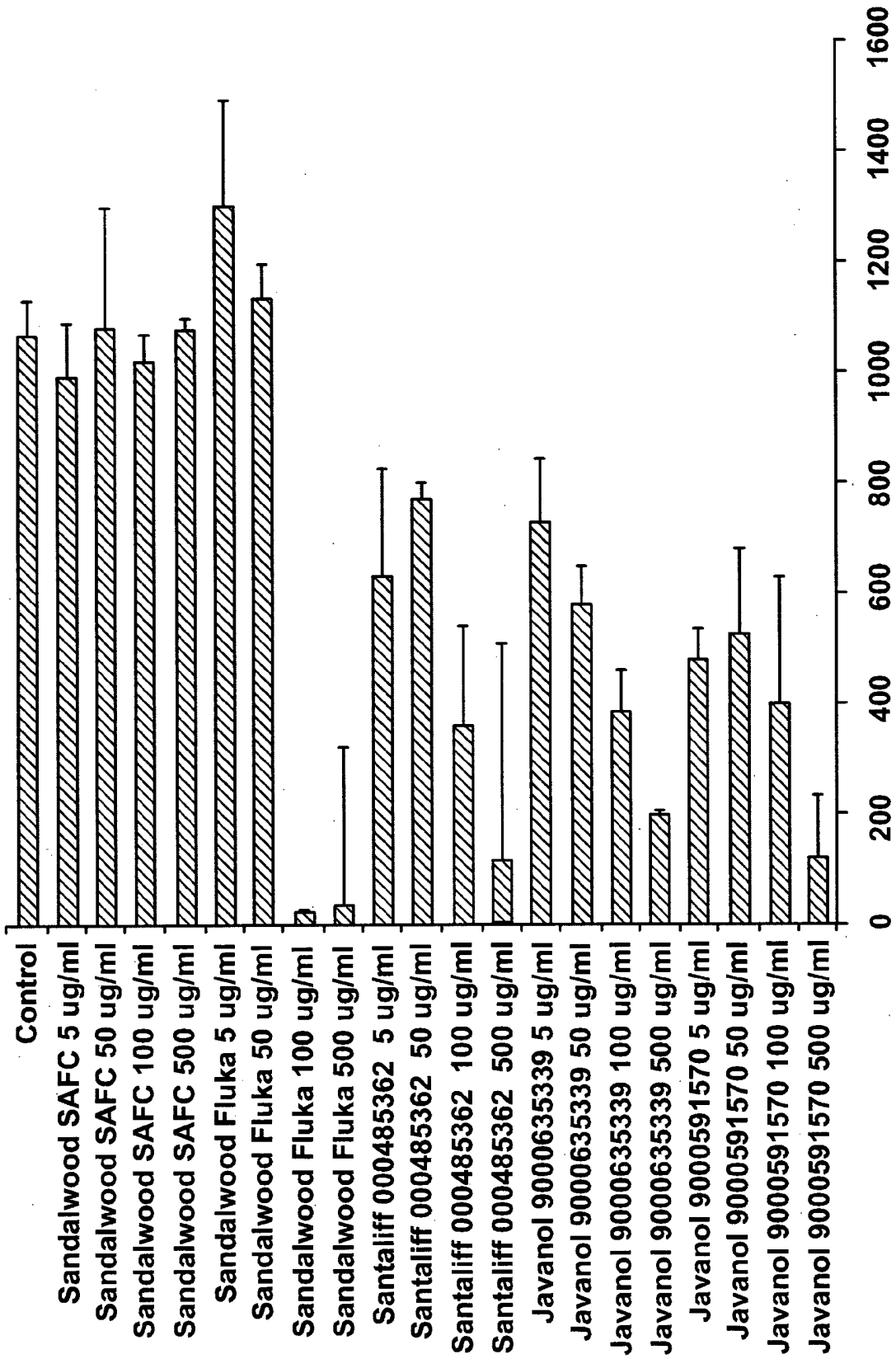

Control
Sandalwood SAFC 5 ug/ml
Sandalwood SAFC 50 ug/ml
Sandalwood SAFC 100 ug/ml
Sandalwood SAFC 500 ug/ml
Sandalwood Fluka 5 ug/ml
Sandalwood Fluka 50 ug/ml
Sandalwood Fluka 100 ug/ml
Sandalwood Fluka 500 ug/ml
Santaliff 000485362 5 ug/ml
Santaliff 000485362 50 ug/ml
Santaliff 000485362 100 ug/ml
Santaliff 000485362 500 ug/ml
Javanol 9000635339 5 ug/ml
Javanol 9000635339 50 ug/ml
Javanol 9000635339 100 ug/ml
Javanol 9000635339 500 ug/ml
Javanol 9000591570 5 ug/ml
Javanol 9000591570 50 ug/ml
Javanol 9000591570 100 ug/ml
Javanol 9000591570 500 ug/ml
0
200
400
600
800
1000
1200
1400
1600
Methane umol/g feed

Fig. 4

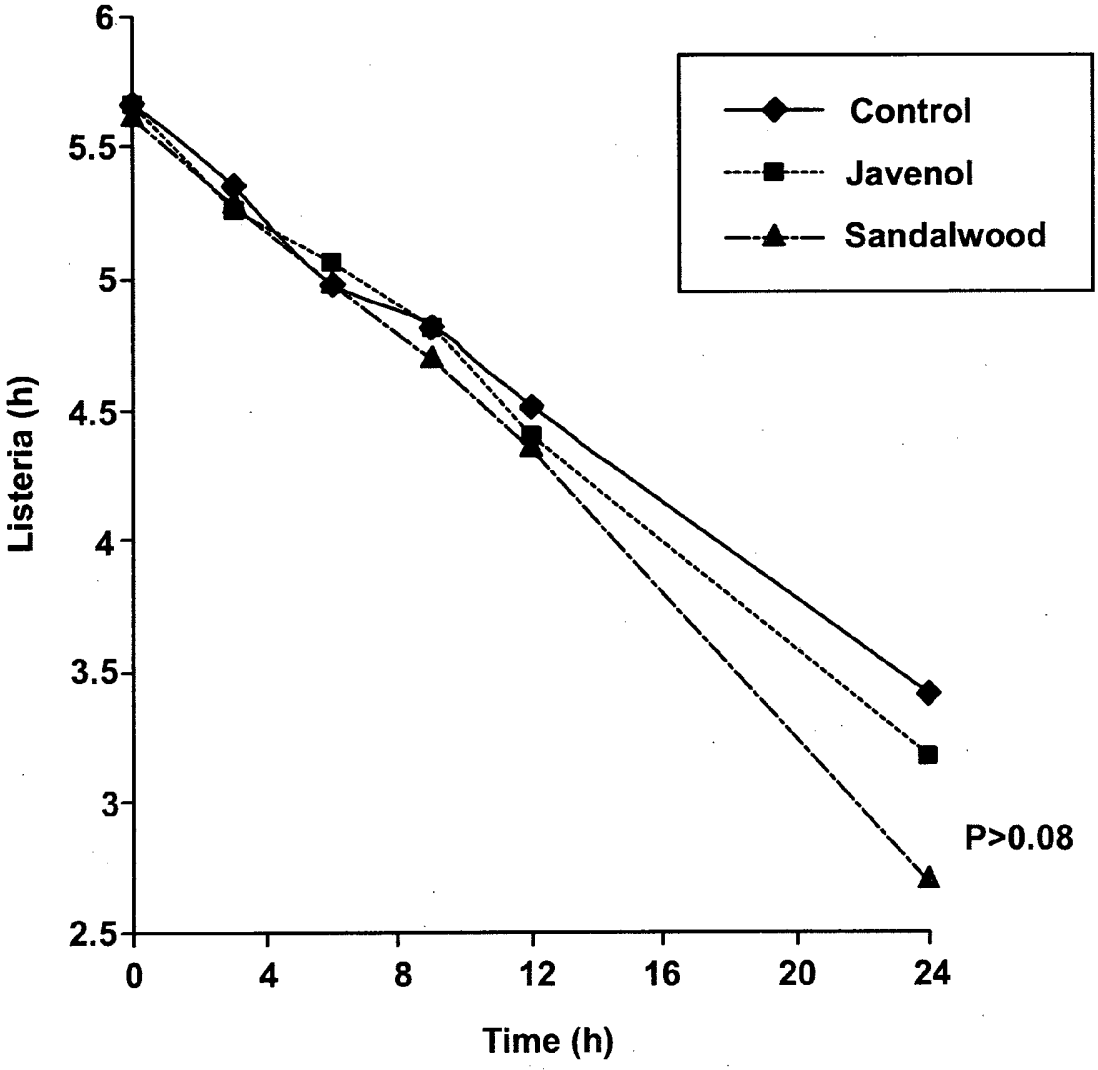
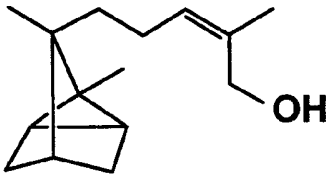
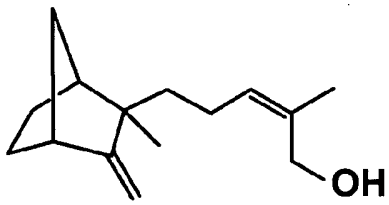

Control
Javenol
Sandalwood
Listeria (h)
6
5.5
5
4.5
4
3.5
3
2.5
0
4
8
12
16
20
24
Time (h)
P>0.08

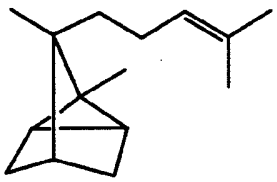
Fig. 5



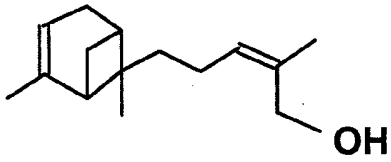
i) α-Santalol



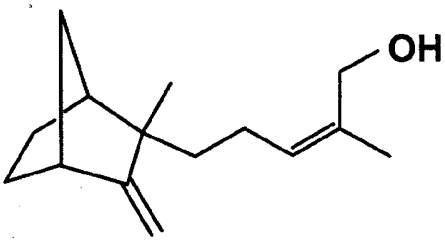
ii) β-Santalol



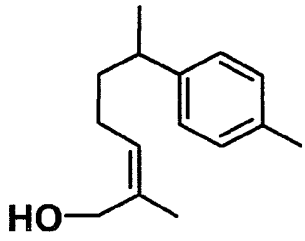
iii) α-Santalene



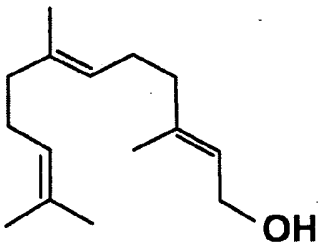
iv) *Z*-α-*trans*-β-Bergamotol



v) *E-cis*,epi-β-Santalol

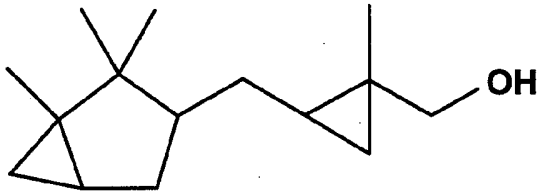


vi) *cis*-Nuciferol

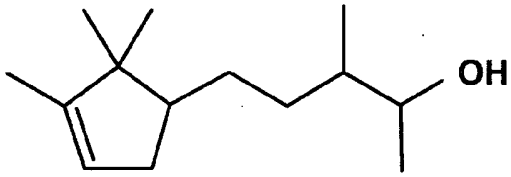


vii) Farnesol (*trans,trans*)

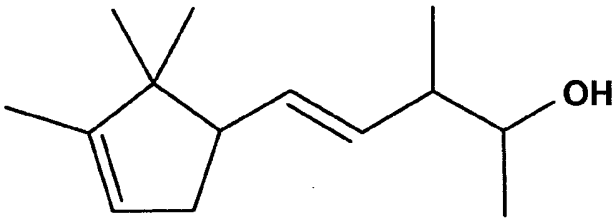
Fig. 6



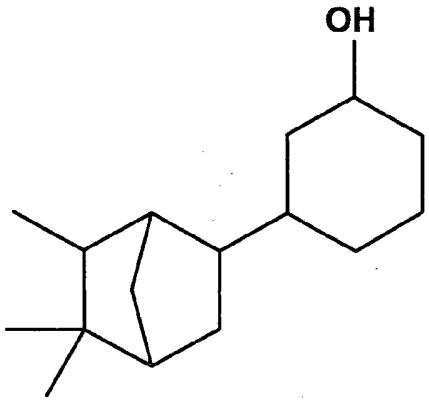
i) Javanol[1]



ii) Sandalore

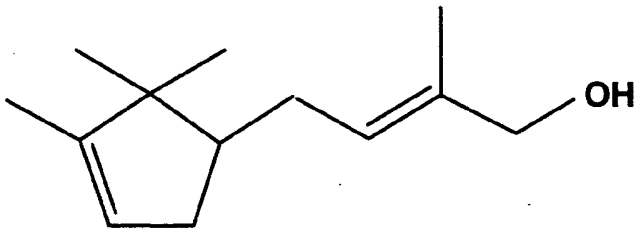


iii) Ebanol

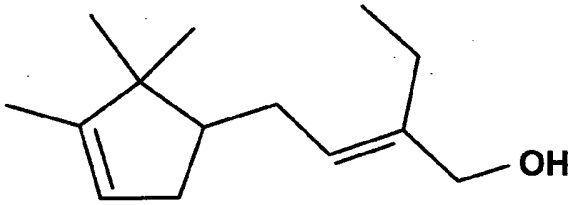


iv) Sandela[2]

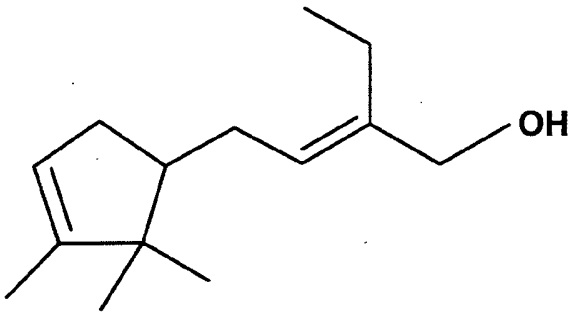
Fig. 7



(i) Santaliff

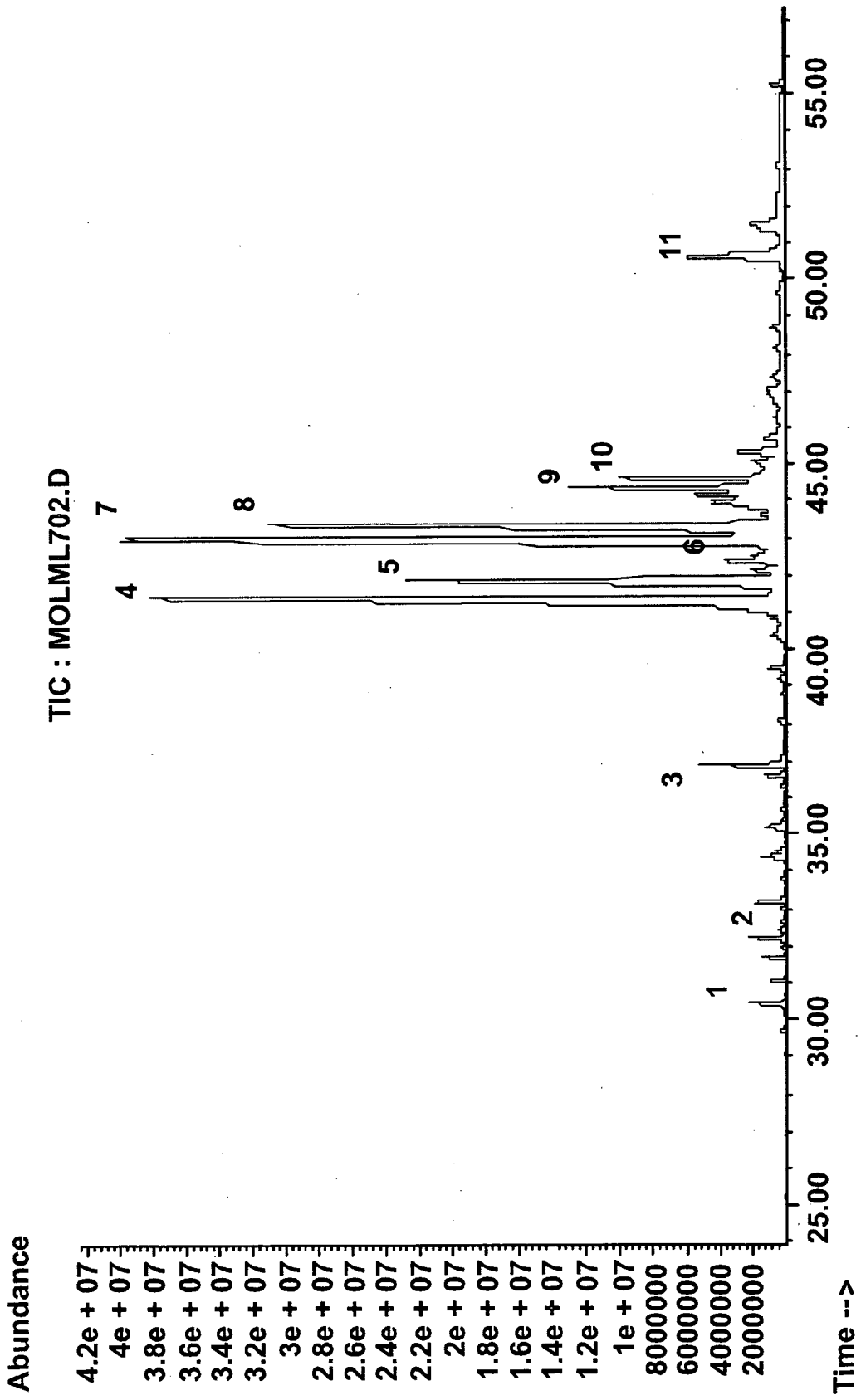


(ii) Bacdanol[1]



(iii) Sanjinol[1]

Fig. 8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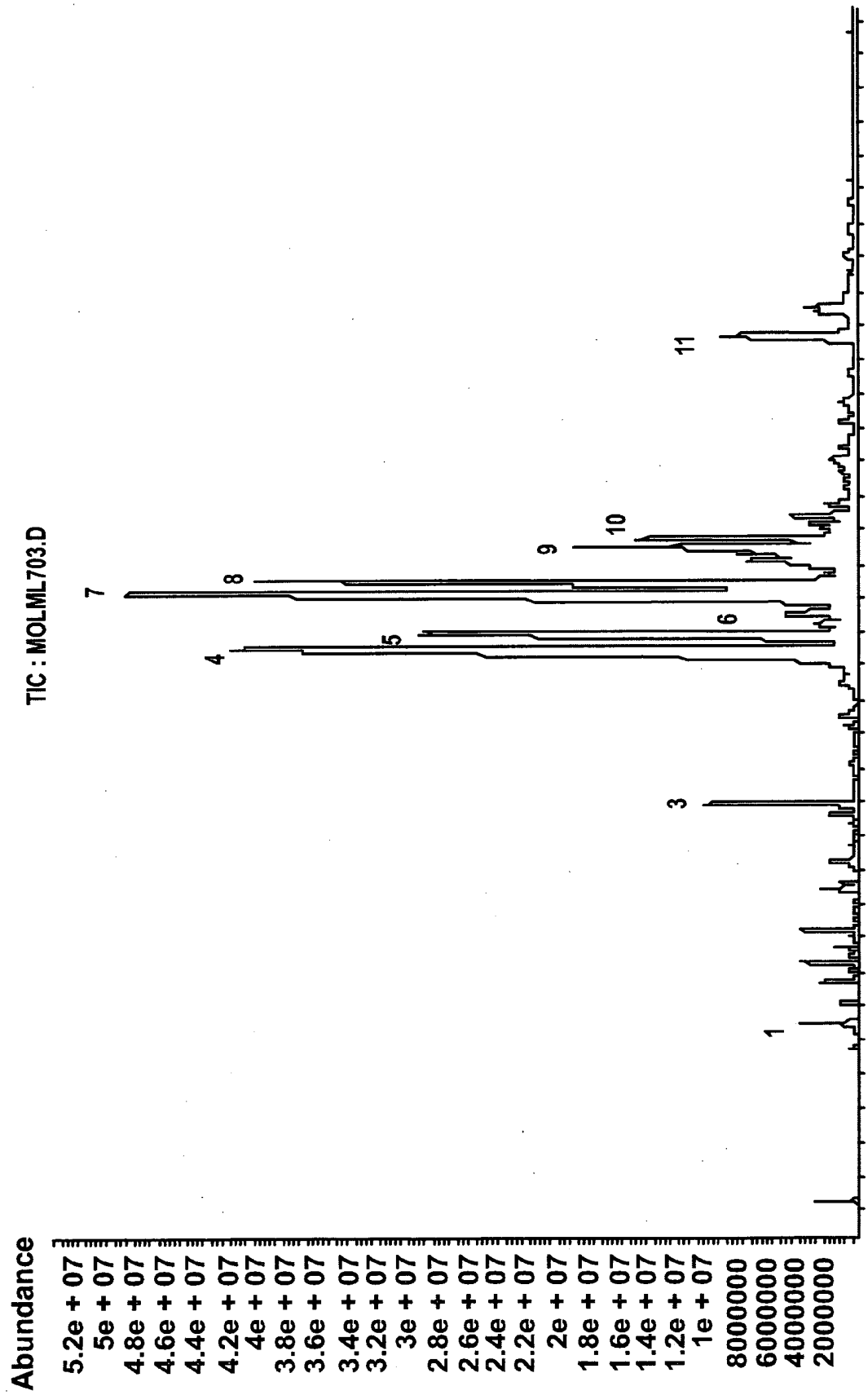
3.6e + 07
3.4e + 07
3.2e + 07
3e + 07
2.8e + 07
2.6e + 07
2.4e + 07
2.2e + 07
2e + 07
1.8e + 07
1.6e + 07
1.4e + 07
1.2e + 07
1e + 07
8000000
6000000
4000000
2000000
25.00
30.00
35.00
40.00
45.00
50.00
55.00
1
2
3
4
5
6
7
8
9
10
11

Fig. 9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4.8e + 07
4.6e + 07
4.4e + 07
4.2e + 07
4e + 07
3.8e + 07
3.6e + 07
3.4e + 07
3.2e + 07
3e + 07
2.8e + 07
2.6e + 07
2.4e + 07
2.2e + 07
2e + 07
1.8e + 07
1.6e + 07
1.4e + 07
1.2e + 07
1e + 07
8000000
6000000
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2000000
Time -->
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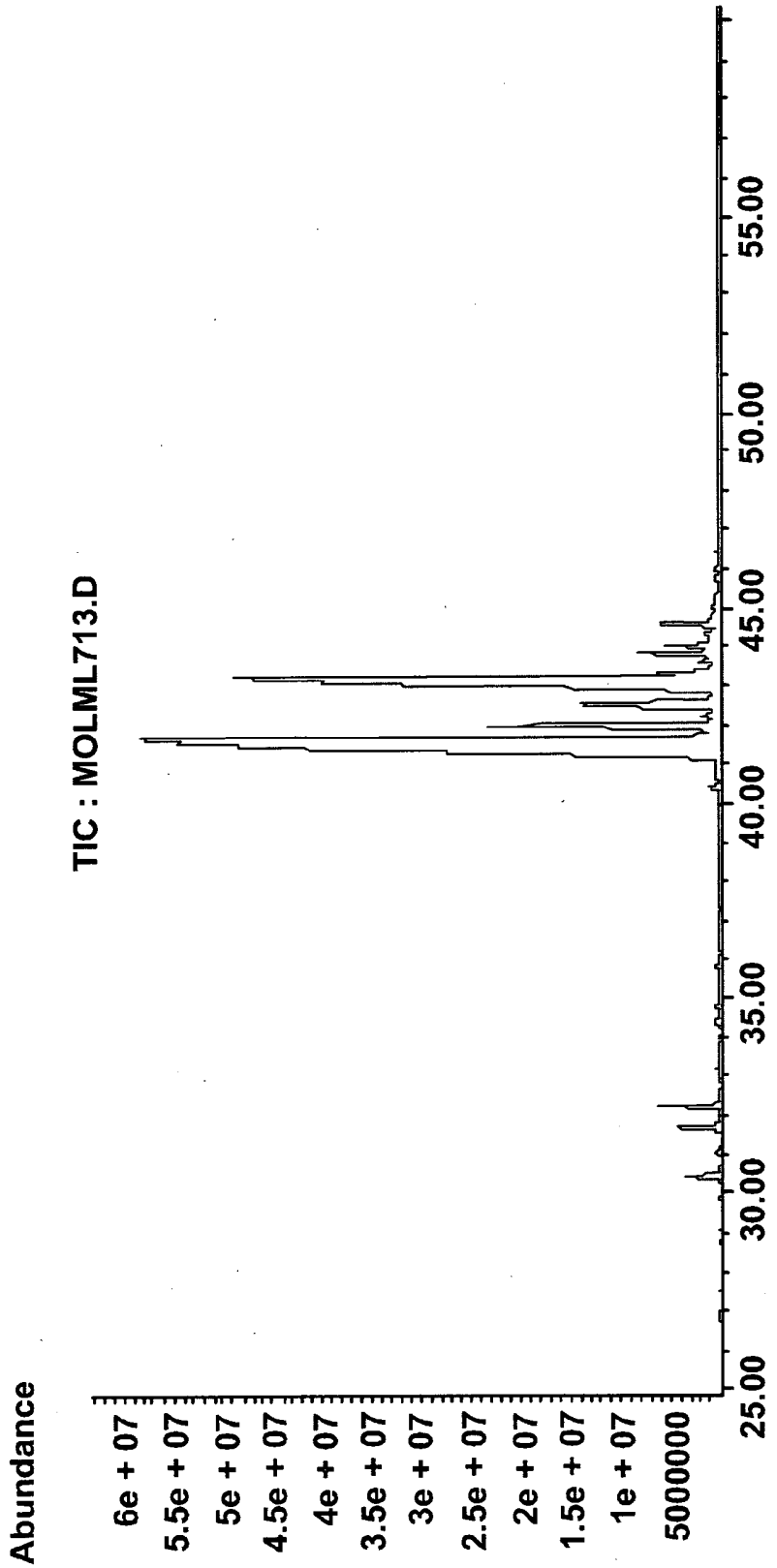
10
11

Fig. 9(cont.)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4e + 07
3.5e + 07
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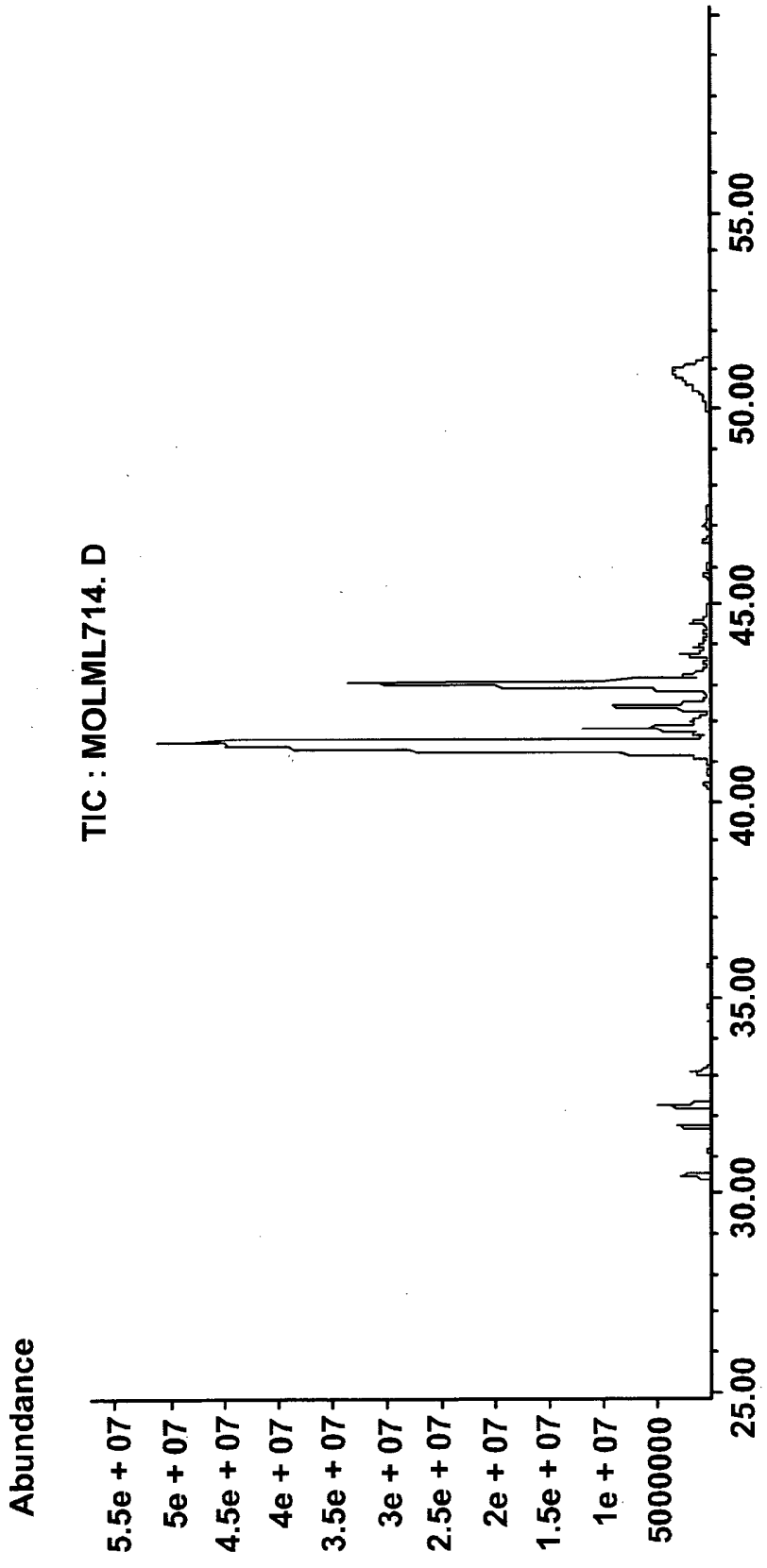
2e + 07
1.5e + 07
1e + 07
5000000
25.00
30.00
35.00
40.00
45.00
50.00
55.00
Time-->

Fig. 10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2.5e + 07
2e + 07
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Time-->
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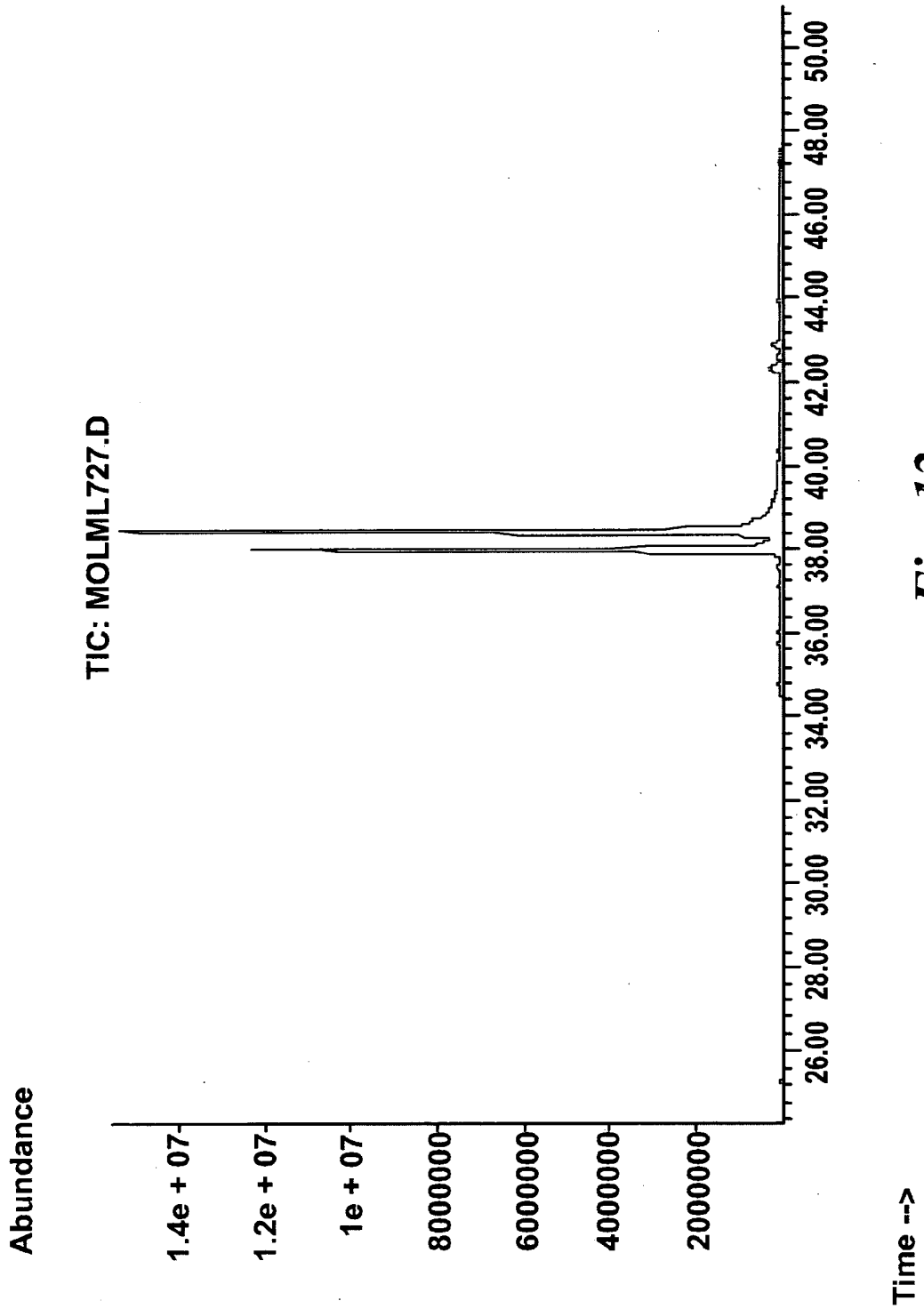
45.00
50.00
55.00

Fig. 11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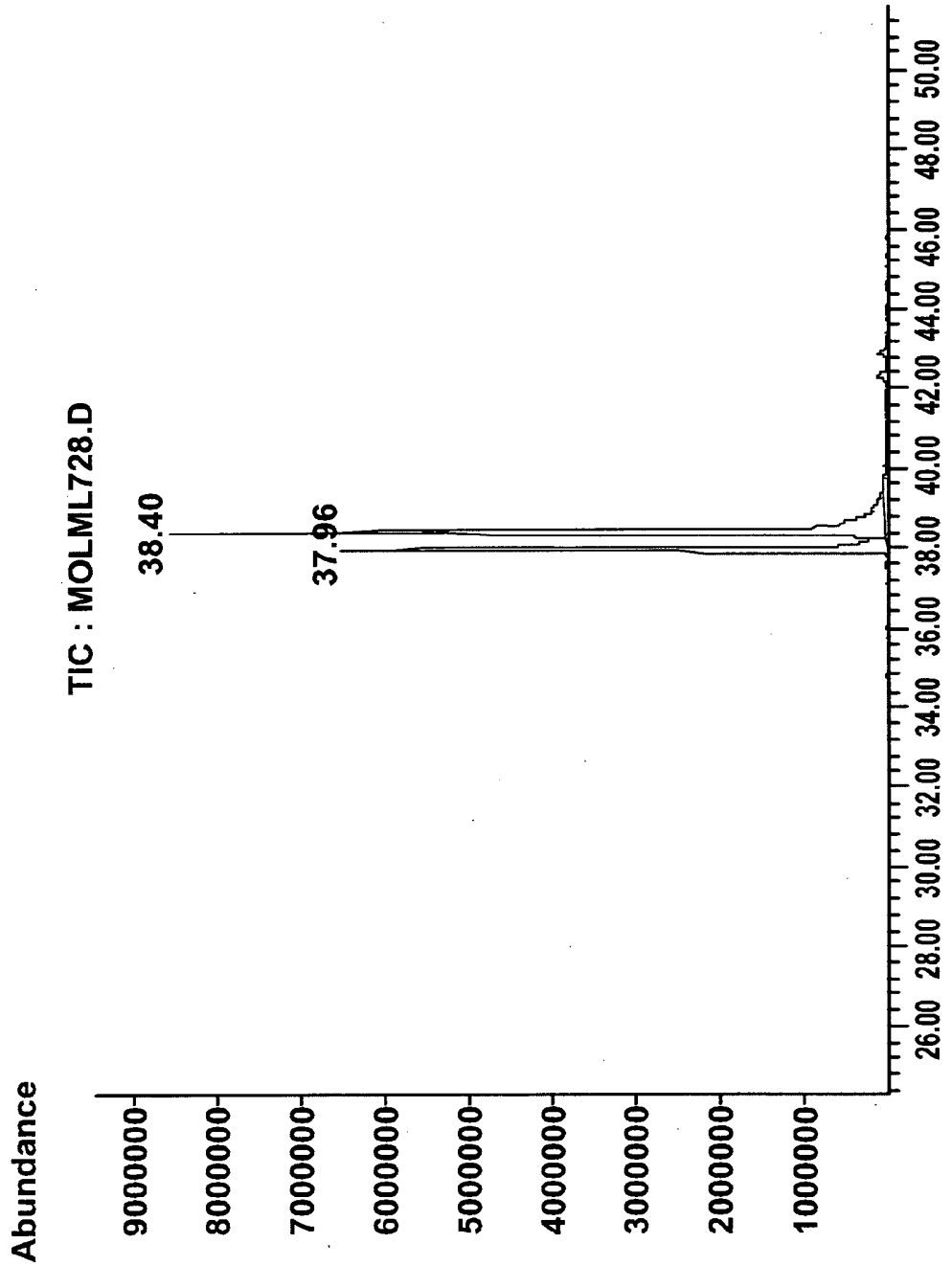
Time -->

Fig. 12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37.96
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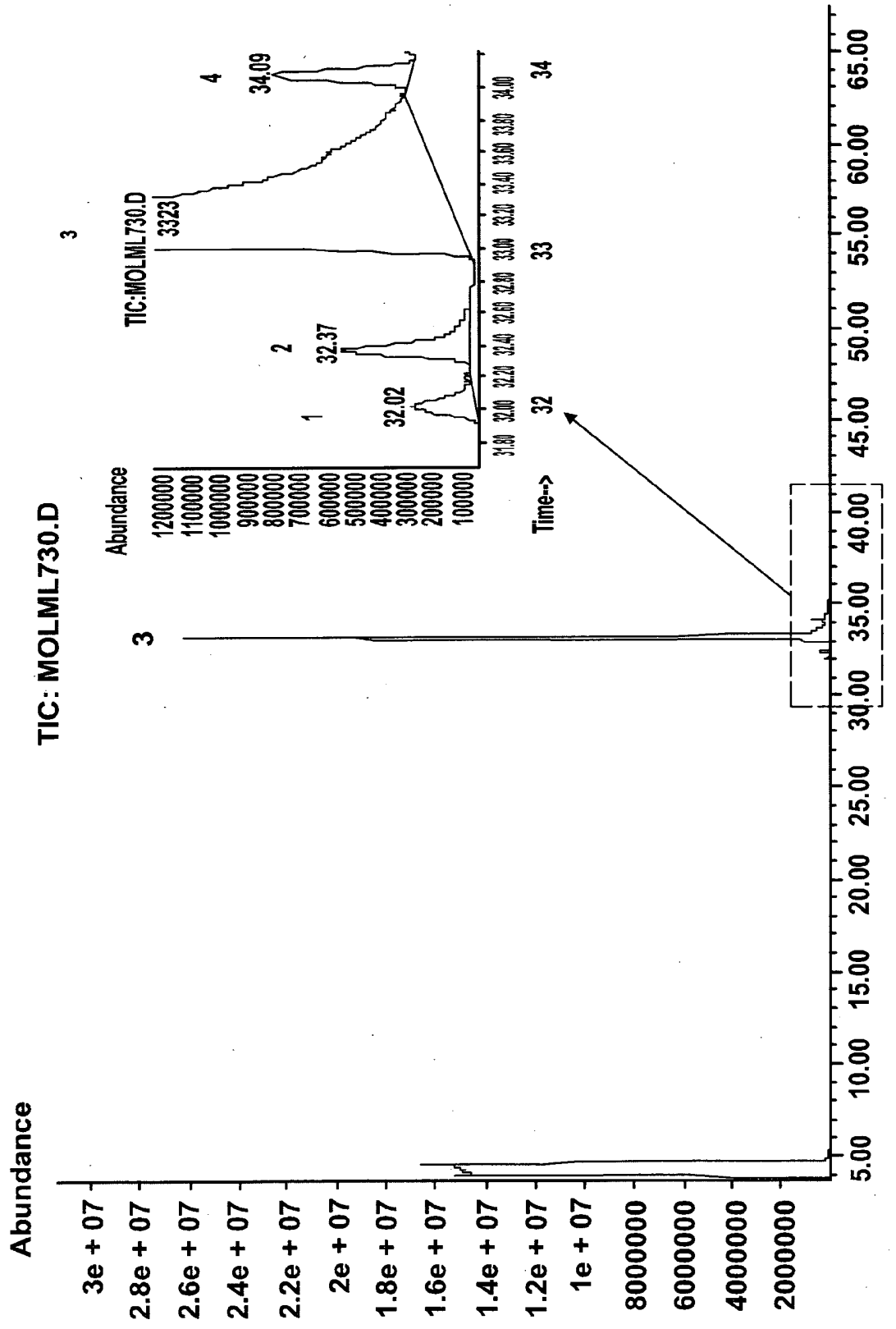
Time -->

Fig. 13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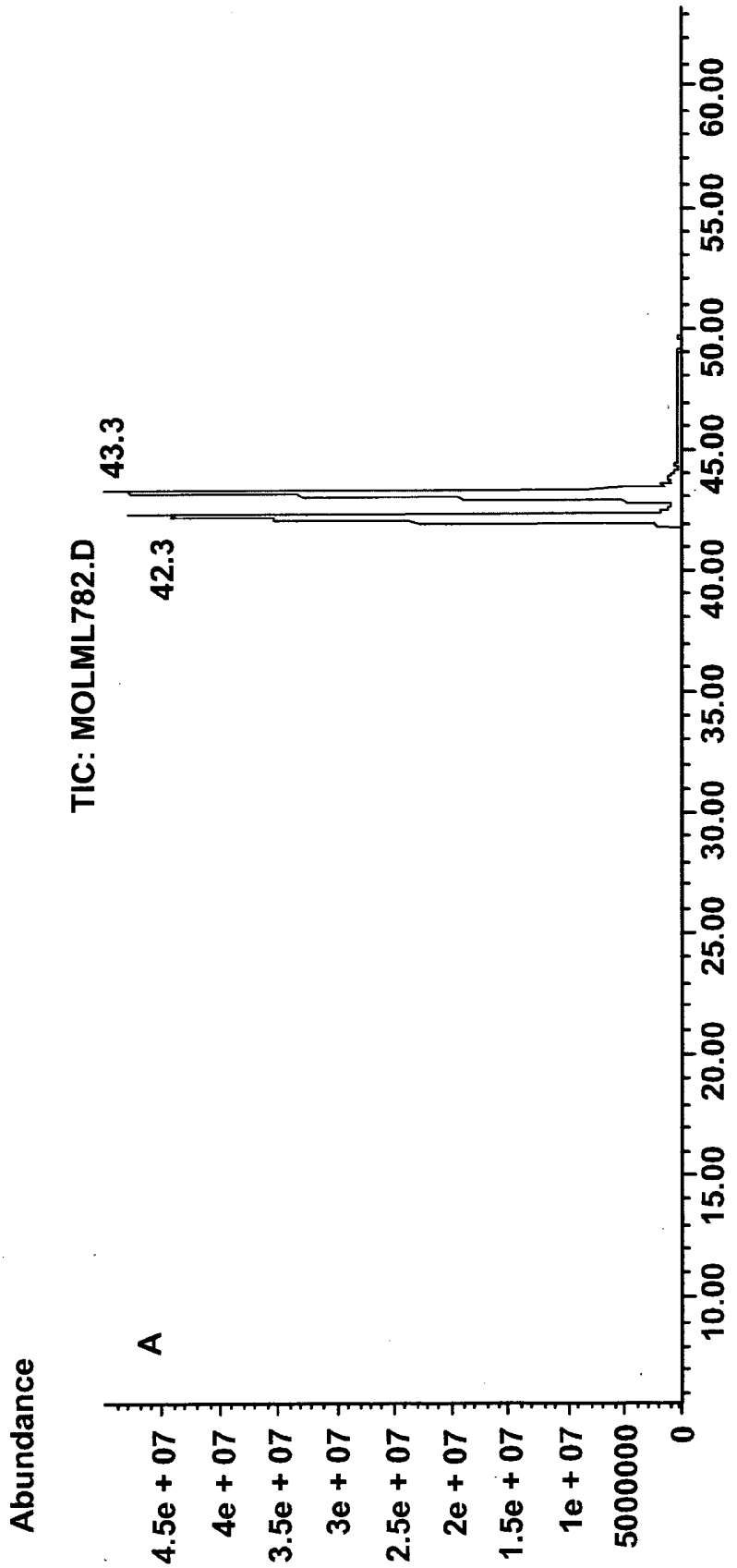
Time-->
TIC:MOLML730.D
32.02
32.37
34.09
3323

Fig. 14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42.3
A
Abundance
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3e + 07
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1e + 07
5000000
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10.00
15.00
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35.00
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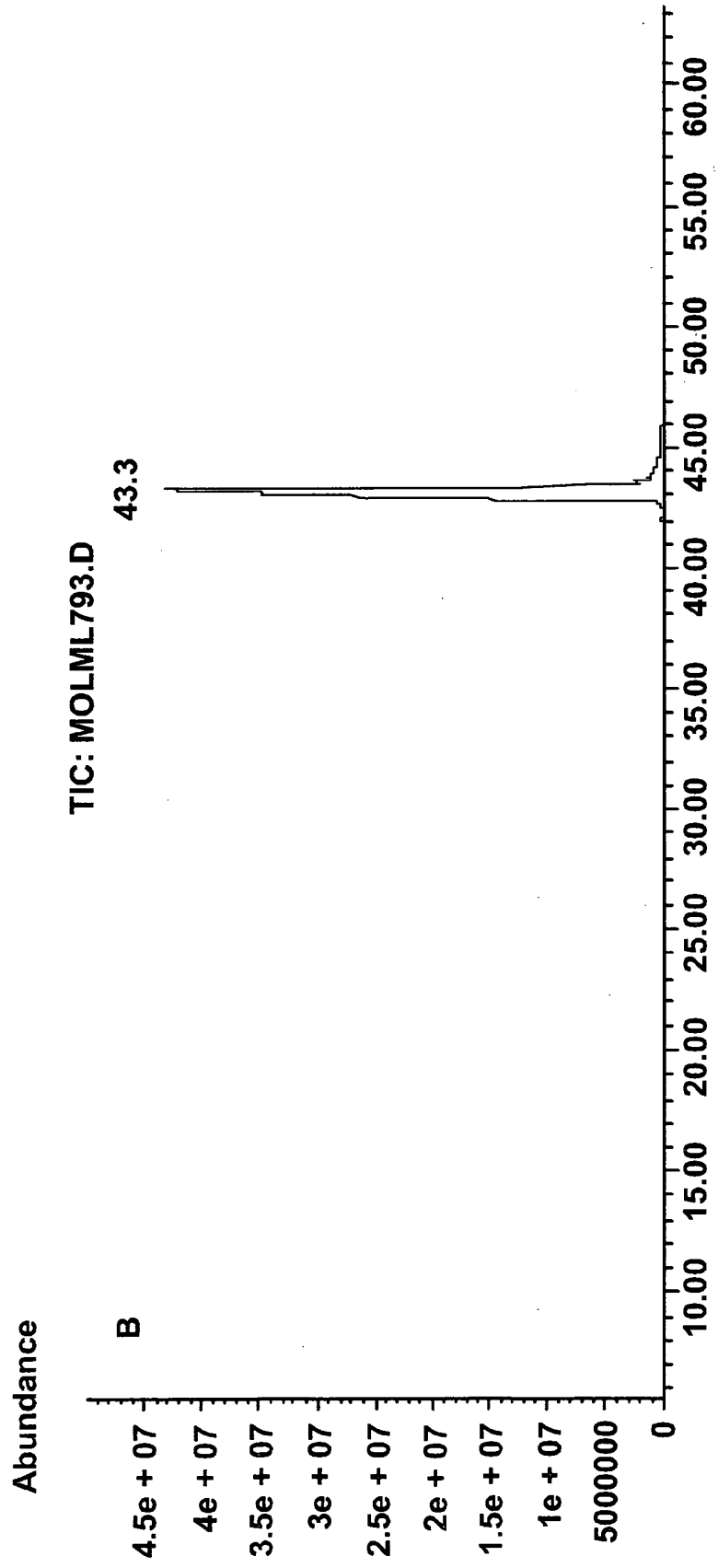
50.00
55.00
60.00
Time -->

Fig. 15


Abundance
B
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3.5e + 07
3e + 07
2.5e + 07
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1e + 07
5000000
0
10.00
15.00
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55.00
60.00
Time -->

Fig. 15(cont.)

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2009/001547

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23K1/16 A23K1/18 A23L1/30 A61K36/185

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A23K A23L A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 2008/148552 A2 (LONZA AG [US]; LONZA AG [CH]; MUSSER ROBERT E [US]; WOODWORTH JASON C) 11 December 2008 (2008-12-11) paragraph [0036]; claims 21,24	1-26, 29-31, 33-34, 36-38
X	DATABASE WPI Week 200216 Thomson Scientific, London, GB; AN 2002-115018 XP002546563 & CN 1 318 383 A (HU J) 24 October 2001 (2001-10-24)	1-17, 33-34, 36-38
Y	abstract	18-26, 29-31

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 September 2009	07/10/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Vermeulen, Stéphane

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2009/001547

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	JP 10 025245 A (KOBAYASHI PHARMA) 27 January 1998 (1998-01-27) abstract	1-17, 33-34, 36-38 18-26, 29-31

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